MONITORING OF THE WINTER 1987 FIELD RELEASE OF GENETICALLY ENGINEERED BACTERIA IN CONTRA COSTA COUNTY

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By
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BIOLOGICAL CONTROL SERVICES PROGRAM

STATE OF CALIFORNIA
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Environmental Monitoring and Pest Management Branch
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EXECUTIVE SUMMARY

The Biological Control Services Program of the California Department of Food and Agriculture conducted a monitoring study of the environmental fate of the genetically engineered microbial pesticide, Frostban®, applied in December, 1987 in Contra Costa County. Genetically engineered bacteria were recovered from air samples collected up to 25 m to the west and north of the experimental strawberry site on the day of application. No bacteria were detected prior to or one day after pesticide application. Frostban® bacteria could be recovered from treated on-site strawberry foliage on every sampling date following application. Vegetation was sampled from 15 to 60 m off-site 22 days before and 1, 3, 7, 14, 22, and 28 days after Frostban® application. Frostban® bacteria were recovered from a limited number of off-site vegetation samples 14 and 22 days post-spray from locations 15 m - 18 m or 60 m to the west or north of the experimental site, respectively. The pattern of detection of genetically engineered bacteria recovered from air and vegetation samples suggests that off-site movement of these bacteria is likely due to drift during pesticide application.

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Disclaimer

The mention of commercial products, their source or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products.

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INTRODUCTION

Genetically engineered microbial pesticides are a new class of products being developed for agricultural use. Frostban® is one of the products currently being tested which utilize genetically engineered strains of *Pseudomonas* species to control frost injury. When applied to crops, these genetically engineered *Pseudomonas* strains are intended to provide an alternative form of frost control through competitive displacement of naturally occurring ice-nucleation active (INA^+) epiphytic bacteria (Lindow, 1982.). These INA^+ bacteria have been shown to be important catalysts of frost injury in plants (Lindow *et*. *al*., 1984). Altered bacteria which lack the ability to initiate frost formation are referred to as INA^- or "ice minus" bacteria.

A field test of Frostban® bacteria was conducted by the biotechnology firm, Advanced Genetic Sciences, Inc. (AGS), on strawberry plants during spring, 1987, in Brentwood, Contra Costa County. Environmental monitoring of these pesticide applications, which constituted the first deliberate release of a genetically engineered microbial product for field testing, was carried out by AGS, the United States Environmental Protection Agency (EPA), and the California Department of Food and Agriculture (CDFA), Environmental Monitoring and Pest Management Branch.

Upon completion of the initial field study, AGS requested and was given approval by CDFA and EPA for an extension of their research authorization and experimental use permit, respectively, in order to conduct an additional field test of Frostban® bacteria on strawberry plants in Brentwood during winter, 1987, and spring, 1988. The CDFA, Biological Control Services Program (BCSP), a unit of the Environmental Monitoring and Pest Management Branch, acting in support of this registration action, initiated a monitoring study of the environmental fate of the genetically engineered microbial pesticide applied in December, 1987. The objectives of this study were:

1. To determine whether genetically engineered Pseudomonas species could be detected over time on vegetation in strawberry fields specified as the release sites for these products in Contra Costa County.
2. To determine whether genetically engineered Pseudomonas species could be detected in air or on vegetation outside of the environmental release sites in order to assess whether there had been off-site movement of these products.

The results of this environmental monitoring study are the subject of this report.

MATERIALS AND METHODS

The study area was located in an agricultural region of Contra Costa County, just east of Brentwood (Figure 1). This location was specified in a research authorization and experimental use permit, approved by the CDFA and EPA, respectively, as the site for release of genetically engineered Pseudomonas species by AGS. The same location was used for similar studies in the spring of 1987.

The experimental site consisted of three adjacent strawberry fields: from north to south, Fields A, B and C (Figure 2). Each field measured 24 m by 43 m, and was comprised of 40 plots, each containing approximately 144 strawberry plants. A bare soil buffer zone 15 m wide surrounded these three fields. In this study, off-site was considered to be the area beyond the 15 m buffer zone.

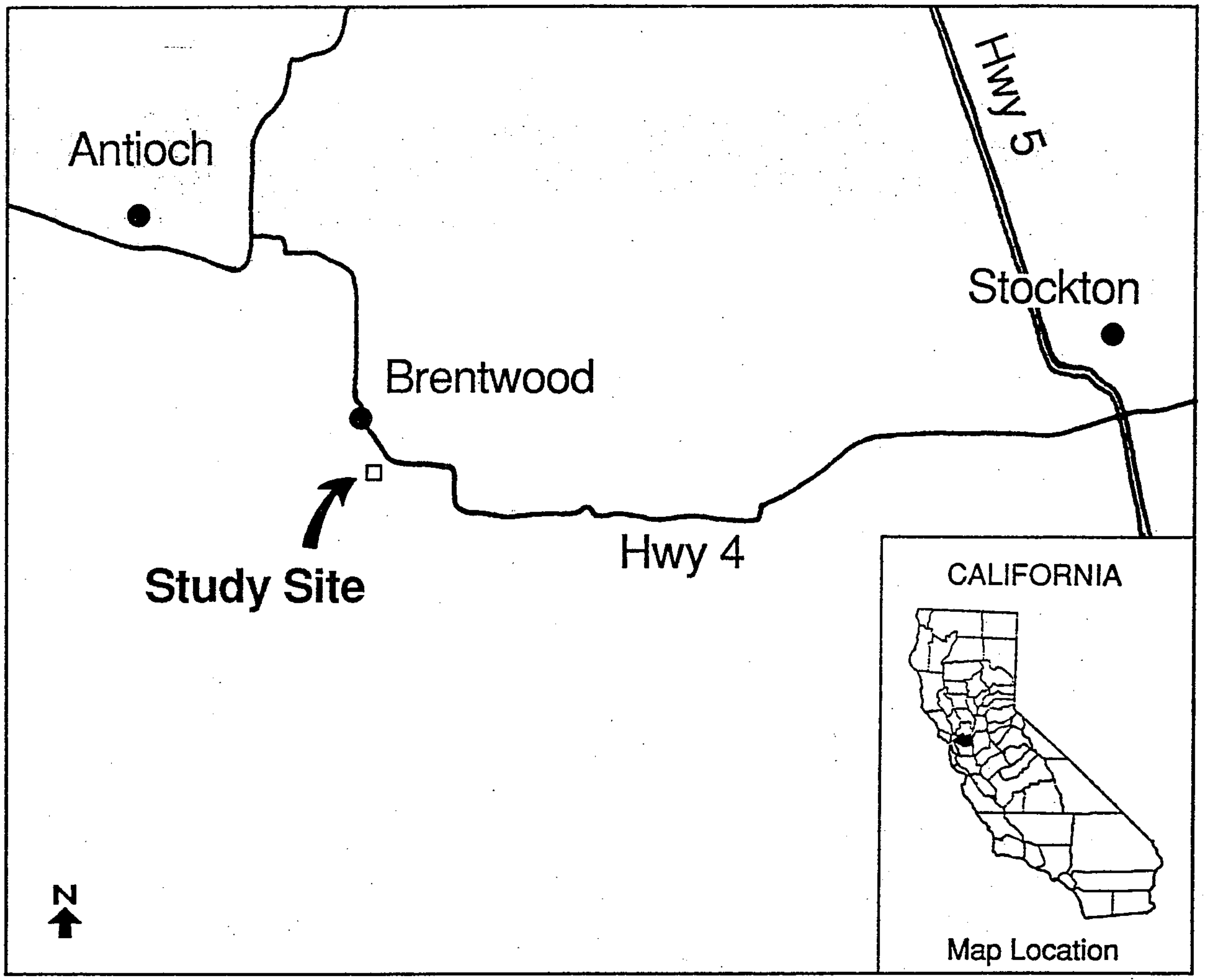

Figure 1. Location of the Frostban® test site in Contra Costa County, California

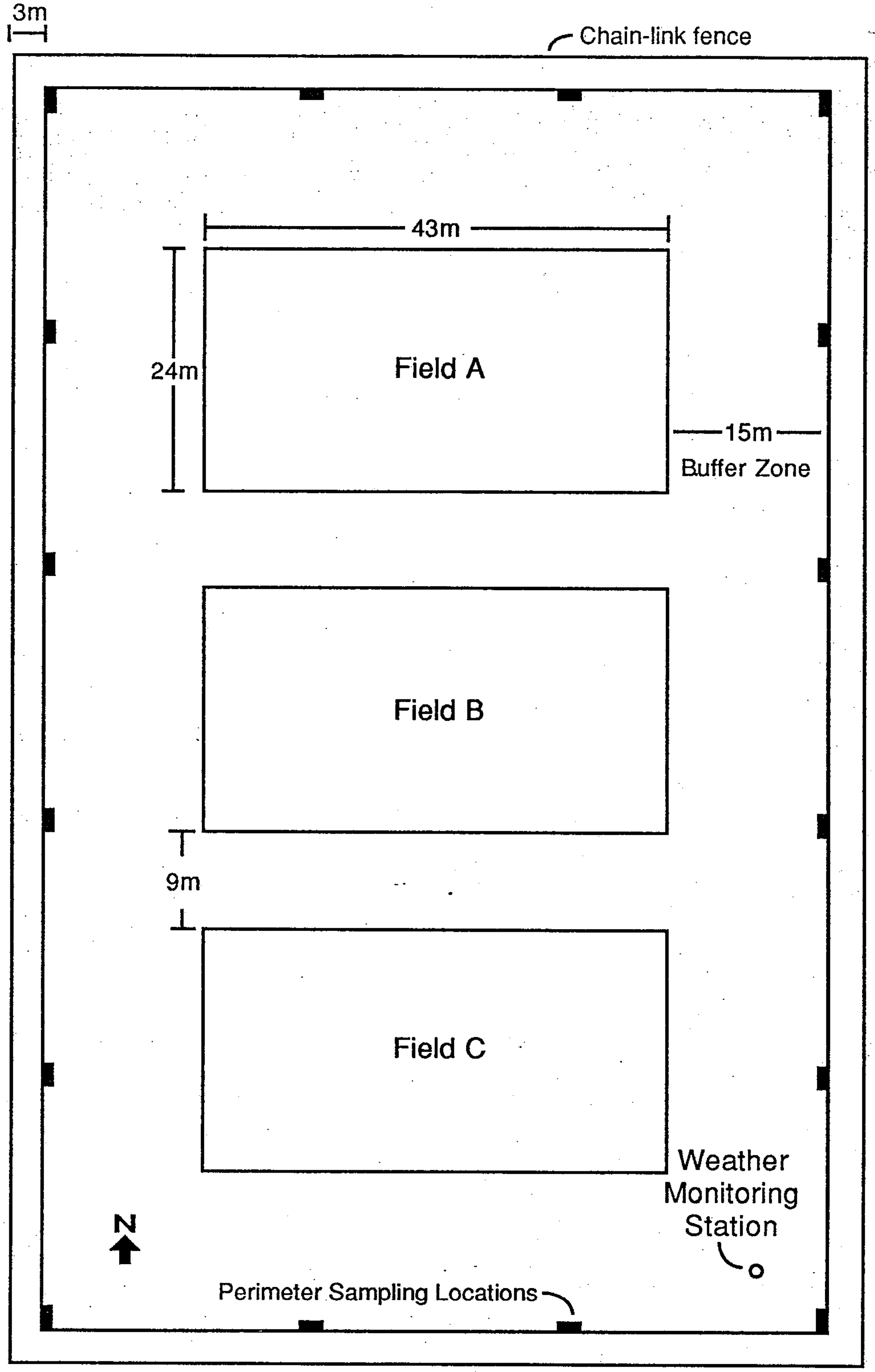

Figure 2. Strawberry field layout for the experimental application of Frostban®. Fields A,B, and C contained 40 plots, each consisting of approximately 144 strawberry plants.

A six foot chain-link fence surrounded the entire study area. This fence was placed 3 m outside of the buffer zone to serve as a barrier to prevent unauthorized access to the study area.

Adjacent to the study site was a fallow cucumber field 60 m from the edge of the strawberry fields to the west. Beyond a dirt access road to the east, was a pear orchard 28 m from the edge of the strawberry fields, and to the north was a second orchard about 60 m from the fields. To the south was a paved road 60 m from the edge of Field C. A large mound of soil adjacent to a sump area, and a narrow drainage trough were located to the northeast.

AGS Experimental Design

The study design for AGS is presented for informational purposes only, since the sole objective of CDFA was to determine the environmental fate of Frostban® while AGS had different test objectives.

Bacterial Strains - Two strains of genetically engineered bacteria in the genus Pseudomonas were used by AGS for these experiments: Pseudomonas syringae, strain RGP36R2; and Pseudomonas fluorescens, strain GJP17BR2. Both strains were selected for resistance to the antibiotic rifampicin. The use of the trade name Frostban® in this report refers to either or both of these strains. These strains were modified by deleting a 400 base-pair segment from the gene which codes for an ice nucleation active protein on the bacterial surface. This deletion renders the bacteria relatively inactive as ice nuclei.

In addition to genetically engineered bacterial strains, naturally occurring ice nucleation active (INA^+) and ice nucleation inactive (INA^-) bacteria were applied as treatments in field experiments.

Study Design - Each of the three strawberry fields was broken into 40 plots. In both Field A and B, 10 treatments were randomly assigned to each of four plots in a complete block design (Tables 1 and 2). No treatments were assigned to Field C. Plots were planted with approximately 144 plants in three double rows each oriented east-west, 1.4 m wide and separated from each other by 1.5 m.

Table 1. List of Treatments for Strawberry Field A.

Treatment Code	Treatment ID	Treatment Dose (cfu/ml)[e]	Time Since Application (hrs)[f]
1	*P. syringae* (INA+)[a]	10^3	0
2	*P. syringae* (INA+)	10^3	0
	P. syringae (GEM)[b]	10^8	0
3	*P. syringae* (INA+)	10^3	0, 48
	Kocide[c]	3.3g/l	4, 44
4	*P. syringae* (INA+)	10^3	0
	P. fluorescens (GEM)[d]	10^8	0
5	*P. syringae* (INA+)	10^3	0
	P. syringae (GEM)	10^8	0
	P. fluorescens (GEM)	10^8	0
6	Nontreated Control	Buffer[g]	0
7	*P. syringae* (INA+)	10^3	48
	P. syringae (GEM)	10^8	0
	P. fluorescens (GEM)	10^8	0
8	*P. syringae* (INA+)	10^3	48
9	*P. syringae* (INA+)	10^3	48
	P. syringae (GEM)	10^8	0
10	*P. syringae* (INA+)	10^3	48
	P. fluorescens (GEM)	10^8	0

a. From known strains of naturally occurring ice nucleation active bacteria.
b. Genetically engineered microorganism (GEM), *P. syringae* RGP36R2
c. Kocide is a commercially available bactericide.
d. Genetically engineered microorganism (GEM), *P. fluorescens* GJP17BR2.
e. Cfu/ml= colony forming units per milliliter.
f. Where two numbers are present, this indicates two separate treatment times.
g. Buffer consisted of a 10 mM $KHPO_4$ solution.

Table 2. List of Treatments for Strawberry Field B.

Treatment Code	Treatment ID	Treatment Dose (cfu/ml)[d]	Time Since Application (hrs)
1	Nontreated Control	Buffer [e]	0
2	_P. syringae_ (GEM)[a]	10^8	0
3	_P. fluorescens_ (GEM)[b]	10^8	0
4	_P. fluorescens_ (INA-) (with Copper resistance)	10^8	0
5	_P. syringae_ (INA-)[c]	10^8	0
6	_P. syringae_ (INA+)	10^3	48
	P. syringae (INA+ with streptomycin resistance)	10^3	48
7	Treatments "2" + "6"		
8	Treatments "3" + "6"		
9	Treatments "4" + "6"		
10	Treatments "5" + "6"		

a. Genetically engineered microorganism (GEM), _P. syringae_ RGP36R2
b. Genetically engineered microorganism (GEM), _P. fluorescens_ GJP17BR2
c. Naturally occurring bacteria.
d. Cfu/ml= colony forming units per milliliter.
e. Buffer consisted of a 10 mM $KHPO_4$ solution.

Applications of Frostban® bacteria and naturally occurring strains of bacteria in the genus Pseudomonas were made to Fields A and B; different combinations were used in each field to address different test objectives. A comparison of the efficacy of individual genetically engineered INA^- strains of Frostban® bacteria against mixtures of naturally occurring bacteria, and a comparison between chemical and biological treatments took place in Field A. Field B was utilized to compare the efficacy of genetically engineered INA^- Frostban® bacteria and two naturally occurring INA^- strains of bacteria in the genus Pseudomonas with and without a challenge presented by naturally occurring INA^+ bacteria.

Dosage - Each treatment of genetically engineered bacteria contained approximately 2×10^8 cells per ml (2×10^{11} cells per liter) in a volume of 10 liters of water. This dosage should result in bacterial populations of 10^6 - 10^7 colony forming units (CFU) per blossom.

Treatment Equipment - All applications to the study plots were made with pressurized (40 psi) backpack sprayers, equipped with three series 80 TeeJet nozzles capable of spraying a single bed in one pass. Sprayers were held close to the ground surface (within approximately .5 m of the plant canopy) to reduce drift.

Air Sampling - Air samples were taken with low volume, high velocity, all glass impingers (AGI), model 7540 (Ace Glass Co.). Air flow through the AGI was 12.6 l/min. Each impinger was initially supplied with 30 ml of .01 molar phosphate buffer solution so that the distance between the tip of the capillary tube and the top surface of the collection fluid was 2 mm.

Thirty AGIs were arranged in five transects (six AGIs per transect), one in each of the four cardinal directions, and the fifth oriented northeast, the direction of the prevailing winds (Figure 3). The AGIs were located at 15, 20, 25, 30, 45, and 60 m from the edge of the experimental strawberry field. All samples were collected at a height of one meter off the ground. The inlet of each AGI was oriented toward the experimental plots.

Background air samples were collected at 15 and 60 m along each of the five transects. In addition, background samples were collected at 20 and 25 m on the northeast transect. All 30 samples were collected on the day of and on the day after application of Frostban®.

Impingers were run for 30 minutes during background sampling. Ice formation, and eventual freezing of the buffer solution in the AGIs was observed during this time period and was the result of low ambient temperature during sampling. To mitigate this problem, the remaining air samplers were run for 30 or 60 seconds and then shut off for either five or six minutes in an alternating manner. Total collection time for air sampling during pesticide application was 10.5 minutes (out of a total of 133 minutes). In order to avoid freezing of collection fluid,

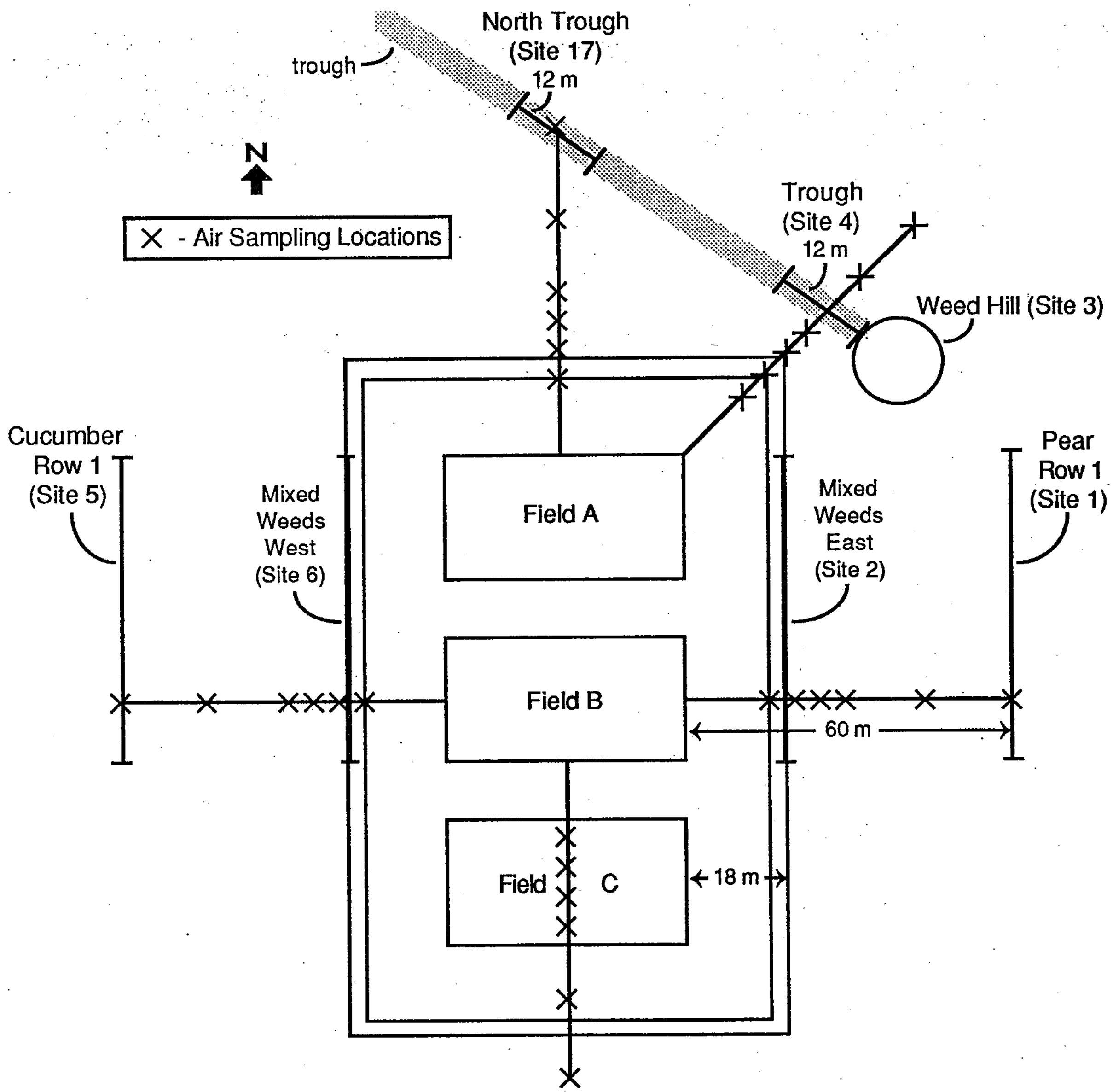

Figure 3. Off-site Vegetation Sampling and Air Sampling Locations at the Frostban® Study Site.

for one day post-spray, the total collection time was five minutes (out of a total of 60 minutes).

To prevent contamination of the collection fluid after sampling, impinger openings were covered with foil or stuffed with cotton and carried to a mobile laboratory. Samples then were immediately processed by sterile filtration of the collection fluid through membrane filters, followed by placement of these membrane filters on selective media. Plates were wrapped with parafilm, covered with foil and transported at ambient temperature to Sacramento for analysis.

On-Site Vegetation Sampling - The CDFA monitoring protocol required vegetation samples from three of the treatments: plots that were treated with genetically engineered P. syringae; plots that were treated with genetically engineered P. fluorescens; and control plots that received only phosphate buffer. Each set of four plots assigned to treatments 2, 4 and 6 in Field A, and 1, 2 and 3 in Field B were sampled as representative of these three treatment types. In both Fields A and B, two composite vegetation samples were collected from each set of plots representing three treatment types, on each sampling date. These vegetation samples were created by randomly choosing two strawberry leaflets from each of the four replicate plots representing a particular treatment, then combining these leaflets in one sample bag, for a total of eight leaflets (eight leaflets approximated 10 grams of plant material) per bulk sample. Samples were taken 22 days prior to the spray, and at 1, 7, 14, 22, and 28 days post-spray.

Although no application of genetically engineered bacteria occurred in strawberry Field C, strawberry samples were taken in this area to assess the movement of Frostban® bacteria to the south of the release sites. Field C was composed of four sections, located at distances of 9-14m (section 1), 15.5-20.5m (section 2), 22-27m (section 3), and 28.5-33.5m (section 4), south of Field B. Two samples of eight randomly selected strawberry leaflets were taken from each of the four sections 22 days prior to spray and at 1, 7, 14, 22, and 28 days post-spray, with the following exceptions: on day 7, 14, 22, and 28 post-spray, only one sample was taken from section 4; on day 22 and 28 post-spray, only one sample was taken from section 3; on day 22 and 28 post-spray, only one sample was taken from section 2.

Additional samples were collected from two locations in Field C, 22 days prior to spray and at 1, 7, 14, 22, and 28 days post-spray. These samples were taken 15 meters south of Field B, approximately 24.4m in, from the east and west edges of the field. Eight leaflets were taken for each sample from an area approximating the 10" X 20" size of the strawberry flats placed around the study area.

Off-Site Vegetation Sampling - Off-site vegetation sampling for this study had two components: mixed weed samples which were taken from 15 to 60 m from the strawberry fields from the weeds growing around the site, and perimeter vegetation samples which were taken from up to 16 strawberry flats placed uniformly around the three strawberry fields, just outside of the buffer zone (Figure 3).

Mixed vegetation samples were taken from the following sites (Figure 3); distances in parentheses were taken from the nearest edge of strawberry fields A and B.

Site 1. Pear Row 1: (60m) Field bindweed (Convolvulus arvensis) was sampled from the first row of pears east of the chain-link fence.

Site 2. Mixed Weeds East: (18m) Mixed weeds were taken from just inside the east side of the chain-link fence.

Site 3. Weed Hill: (30m) Mixed weed samples were taken from the perimeter of a mound of soil at the northeast corner of the study area.

Site 4. Trough: (30m) Mixed weeds were taken from both banks of a 12 m section along a drainage ditch extending in a northwesterly direction from the weed hill.

Site 5. Cucumber Row 1: (60m) Mixed weeds were sampled from a cucumber field to the west that had been harvested but not yet disked.

Site 6. Mixed Weeds West: (18m) Mixed weeds were taken from just inside the west side of the chain-link fence.

Site 17. North Trough: (60m) Mixed weeds were sampled from the drainage ditch (see #4) along a 12 m length. The farthest point on the north AGI transect was the midpoint of this sample. Weeds were taken from the two banks of the ditch.

At least one mixed vegetation sample was collected 22 days prior to spray and at 1, 7, 14, 22, and 28 days post-spray, except for site 17 where sampling began 7 days post-spray. Replicate samples were taken at site 2, on day 7, 14, 22, and 28 post-spray. At sites 6 and 17, on days 22 and 28 post-spray, replicate samples were taken, after preliminary results from air sampling indicated potential for movement to the west and north of the experimental strawberry fields.

Perimeter vegetation samples were taken 22 days prior to spray and at 1, 3, 7, 14, 22, and 28 days post-spray. Perimeter vegetation samples consisted of 15 samples per sampling date, one for each strawberry flat, with eight randomly selected leaflets from each strawberry flat comprising one sample, except at background and one day post-spray sampling intervals when 16 samples were collected. After one day post-spray, plants in one of the flats became desiccated and further samples could not be taken, therefore, an additional sample was taken at Site 2.

Samples were stored in coolers until they could be transported to Sacramento where they were refrigerated at 4°C until delivered to the CDFA Analysis and Identification (A&I) Plant Pathology Laboratory. All samples were weighed within 12 hours after collection.

Quality Control

For each week of sampling, including background, six quality control samples were created and interspersed with field samples prior to transport to the A&I laboratory. Strawberry leaves similar to those at the study site were inoculated with one or both species of genetically engineered bacteria.

Weather Monitoring

Continuous wind speed and direction data were collected at approximately 2 m using a Weather Measure Meteorological Station located in the southeast corner of the study area (Figure 2). Collection of weather information began 1.5 h prior to the spray event and continued for 22.5 h, through the collection of air samples one day after microbial pesticide application.

Additional weather data was collected from November 15, 1987 to January 15, 1988 in Brentwood, California, by the California Department of Water Resources.

Laboratory Procedures

Preparation of Samples - All plant material was transferred to sterile half-gallon vessels containing 10 ml of sterile distilled water per gram of tissue, sealed and placed on a reciprocating shaker at 250 rpm for 2 h. A 10 ml aliquot of wash buffer was saved and used to inoculate selective media as a non-concentrated sample. A 50 ml aliquot of wash buffer was centrifuged at 7500 rpm for 10 minutes to concentrate the bacteria. Excess wash water was decanted and the pellet resuspended in the remaining 5 ml. Original wash water (45 ml) and concentrated suspension were used to inoculate selective media containing cycloheximide, rifampicin, and $CuSO_4$ (Appendix I). For each culture plate, 0.1 ml of wash water was uniformly spread to inoculate the surface of selective media. Plates were then sealed with parafilm, inverted and incubated on a laboratory bench at room temperature.

Evaluation of Samples - After 48 to 72 hours at room temperature, culture plates were rated for growth of Frostban® bacteria according to the number of individual colonies on the selective media as follows: (0) indicated 0 colonies, (1) indicated a range of 1-5, (2) indicated a range of 6 to 25, (3) indicated a range of 26 to 100, and (4) indicated the plate was overgrown with greater than 100 colonies.

Using the sample preparation procedure summarized above, each individual colony on selective media represented 10 colony forming units (CFU) per milliliter of unconcentrated wash water (10^2 CFU per gram of leaf tissue) or 1 CFU per milliliter of concentrated wash water (10 CFU per gram of leaf tissue).

All plates with bacterial growth were illuminated under ultraviolet light to detect the presence of any Pseudomonas type bacteria similar to the Frostban® organisms. Distinct fluorescent colonies were identified, circled, examined for morphological type and transferred to plates of King's medium B (see Apendix I) for further characterization. A maximum of five colonies of each morphological type were characterized in this way for each sample analyzed.

AGS utilized a naturally occurring rifampicin resistant ice nucleation inactive strain of P. fluorescens which possessed distinctive characteristics when grown on media containing copper. Colony morphology was used to distinguish this naturally occurring strain of P. fluorescens from genetically engineered P. fluorescens with which it was similar in all other respects. Distinct colony morphology characteristics were: for copper resistant P. fluorescens, a large colony with a mostly smooth edge, appearing domed initially, and later becoming flat and spreading; for genetically engineered P. syringae, a medium colony which developed a convoluted edge and became crater-like as it developed a distinct cloudy white cast; and for genetically engineered P. fluorescens, a small colony usually with a smooth edge which sometimes became domed and viscous.

After 48 hours, isolates on King's B medium were tested for the oxidase and arginine reactions and ice nucleation activities (Appendices II-IV). Bacteria from field samples and laboratory quality control samples were transferred to Luria broth prior to delivery to Dr. Schroth at U.C. Berkeley for gene probe analysis (Appendix V).

A positive determination of genetically engineered P. syringae was made if the following criteria were met: growth on rifampicin, fluorescence, oxidase negative, arginine negative, negative for ice nucleation, and positive identification as genetically engineered Pseudomonas syringae through gene probe analysis. A positive determination of genetically engineered Pseudomonas fluorescens was made if the following criteria were met: growth on rifampicin, fluorescence, oxidase positive, arginine positive, negative for ice nucleation activity, and positive identification as genetically engineered Pseudomonas fluorescens through gene probe analysis. Isolates that failed to meet these criteria were considered to be naturally occurring organisms.

Quality Control Samples - Isolates of genetically engineered P. syringae and P. fluorescens used as controls for this procedure were grown on King's B medium for 36 to 48 h at ambient laboratory temperature (70-75^{O}F). Individual colonies were then selected and used to inoculate 50 ml of nutrient broth amended with 50 mg/l rifampicin, contained in 250 ml Erlenmeyer flasks wrapped with aluminum foil. Flasks were placed on a shaker and rotated at 200 rpm for 36 to 48 h at ambient laboratory temperatures.

A 10 ul aliquot from the 36 to 48 h nutrient broth culture was transferred to a flask containing 100 ml of sterile peptone buffer with a bacterial suspension containing one of the genetically engineered Pseudomonas species.

After mixing for 15 seconds with a vortex mixer, this diluted bacterial suspension was transferred to a sterile Chromist® jar. Each quality control sample was composed of between 10 and 11 grams of strawberry leaflet material (approximately eight leaflets), collected from strawberry plants maintained in a greenhouse in Sacramento. Using a Chromist® sprayer, both the upper and lower surfaces of the leaflets were sprayed with inoculum to runoff. Following inoculation of leaflets, the biohazard hood was cleaned thoroughly with 70% alcohol between each sample.

Statistical Analysis

To determine if there was a difference between sampling times and places, in the probability of recovering fluorescent, non-genetically engineered rifampicin resistant bacteria, logistic regression analysis was performed using the CATMOD procedure (SAS, 1986) with the presence or absence of these bacteria in each sampling location for each sampling date as the dependent variable, and the sampling date, general sampling area (off-site weeds, strawberry field C, or perimeter flats containing strawberry plants), and the date x area interaction as the independent variables.

RESULTS

Weather Monitoring

The mean wind speeds measured for the 1.5 hr period before microbial pesticide application, for the period during spray, for the 20 hr period following application and for the period from 20 to 21 hrs post-spray were 1.05 (0.63) mph, 3.10 (0.62) mph, 1.90 (2.30) mph, and 12.35 (1.61) mph, respectively. The first, second and fourth abovementioned time intervals coincide with air sampling periods. During the period of background air sampling, wind direction was from the south, southeast or southwest. Wind direction during the period of Frostban® application was from the south or southeast (to the north or northwest) (Figure 4). For the 20 h period between the last two air sampling events, wind from all directions was represented although wind direction was less often from the north, northeast or northwest (Figure 5). Winds were entirely from the southeast during 20 to 21 hrs after application (Figure 5).

Air Samples

Genetically engineered P. fluorescens but not P. syringae was detected in air samples collected during Frostban® application from two out of five air monitoring transects. These two transects, to the north and west of the release sites (Figure 3), were oriented in the same direction as wind movement during the spray events. During application, air concentration of Frostban® bacteria ranged from 23 to 38 CFU/M^3 of air at 15 m and 8 CFU/M^3 of air at 20 to 25 m west of the strawberry release site while air concentration ranged from 23 to 38 CFU/M^3 of air 15 and 25 m north of the site. No genetically engineered bacteria were detected in air samples prior to or one day after Frostban® application.

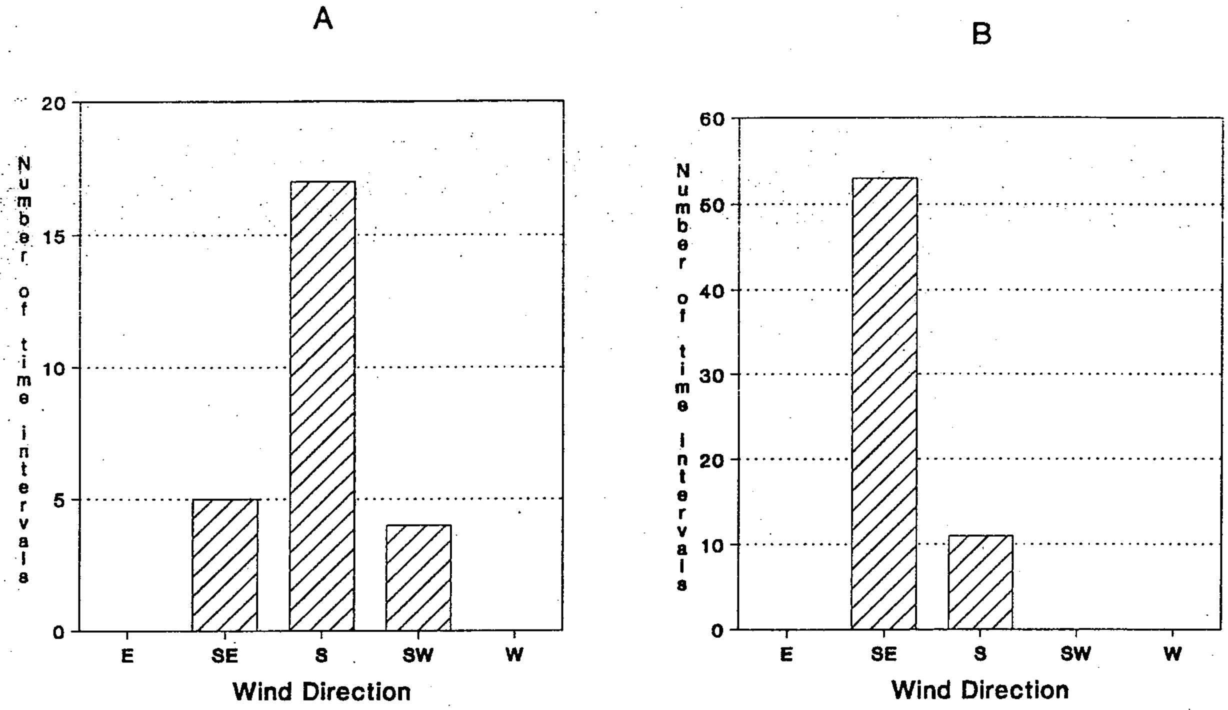

Figure 4. Number of two-minute time intervals where average wind direction was oriented in one of eight ordinal directions during the period before (A) and during (B) Frostban® application.

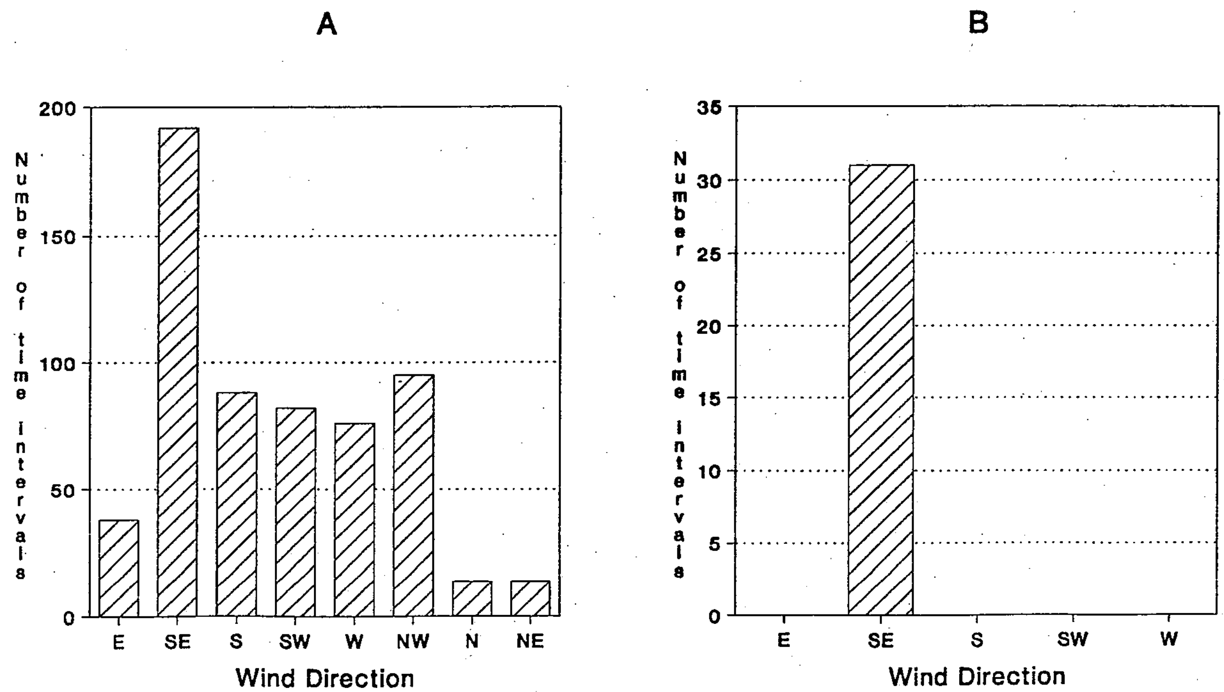

Figure 5. Number of two-minute time intervals where average wind direction was oriented in one of eight ordinal directions during the 20 h. period after Frostban® application (A), and during air sampling from 20 to 21 h. post-spray (B).

Genetically engineered Frostban® bacteria were not detected in air samples at any distances greater than 25 m from the edge of the treated fields.

Vegetation Samples

On-Site Samples - No genetically engineered bacteria were recovered from foliage samples collected before Frostban® application (Appendix VII). Frostban® bacteria were not detected in either background or post-spray strawberry foliage collected from on-site locations in strawberry Field C (which was not treated in this experiment). However, following microbial pesticide application, for every sampling date, genetically engineered bacteria were detected on strawberry foliage collected from treated plots (Figures 6 and 7). One day after Frostban® application, rifampicin resistant fluorescent bacteria were recovered in numbers greater than 10^4 CFU/g strawberry leaf tissue from treated plots. It was not possible, given the method employed in estimating the number of rifampicin resistant bacteria in samples, to determine more precisely the number of CFU/g leaf tissue above the upper limit of resolution of 10^4 CFU/g. Rifampicin resistant, fluorescent Pseudomonads could include genetically engineered or wild Pseudomonas species as well as other bacteria capable of growing in selective media and fluorescing under appropriate test conditions.

One week after Frostban® application, levels of rifampicin resistant fluorescent Pseudomonads remained above the upper limit of resolution of 10^4 CFU/g leaf tissue in 3 out of 4 of the plots treated with Frostban® bacteria. The number of rifampicin resistant fluorescent Pseudomonads detected in samples from treated plots remained between 10^3 and 10^4 CFU/g leaf tissue for the duration of the monitoring period (Figures 6 and 7).

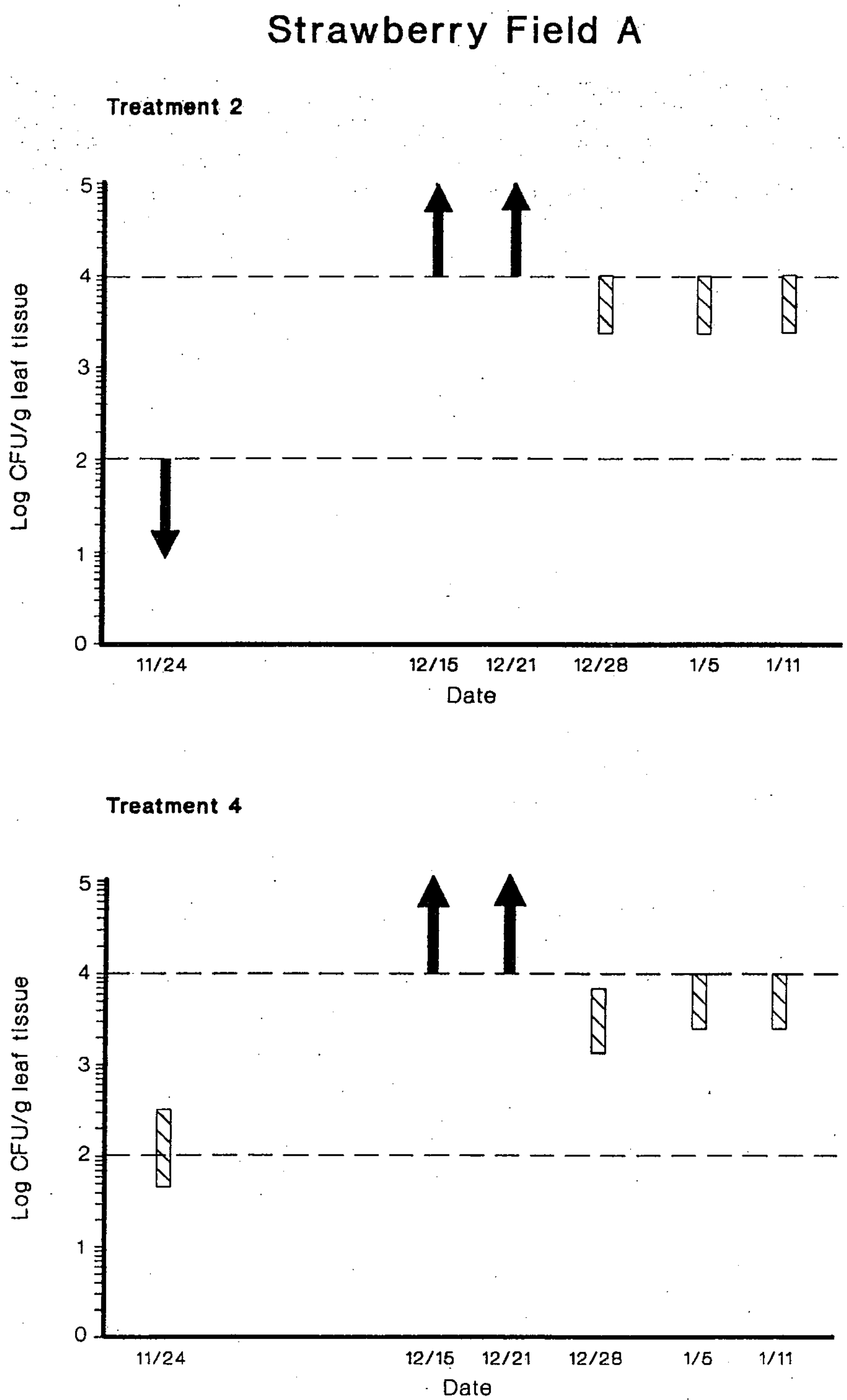

Figure 6. Calculated mean range of abundance of rifampicin resistant, fluorescent bacteria on strawberry leaflets collected from treatment plots during the Frostban® study. Frostban® treatment occurred 12/14/87. Solid arrows indicate values below or above the resolution of the isolation method. Hatched bars indicate the calculated mean range of abundance for bacteria detected between these upper and lower boundaries. Description of treatments 2 and 4 is given in Table 1.

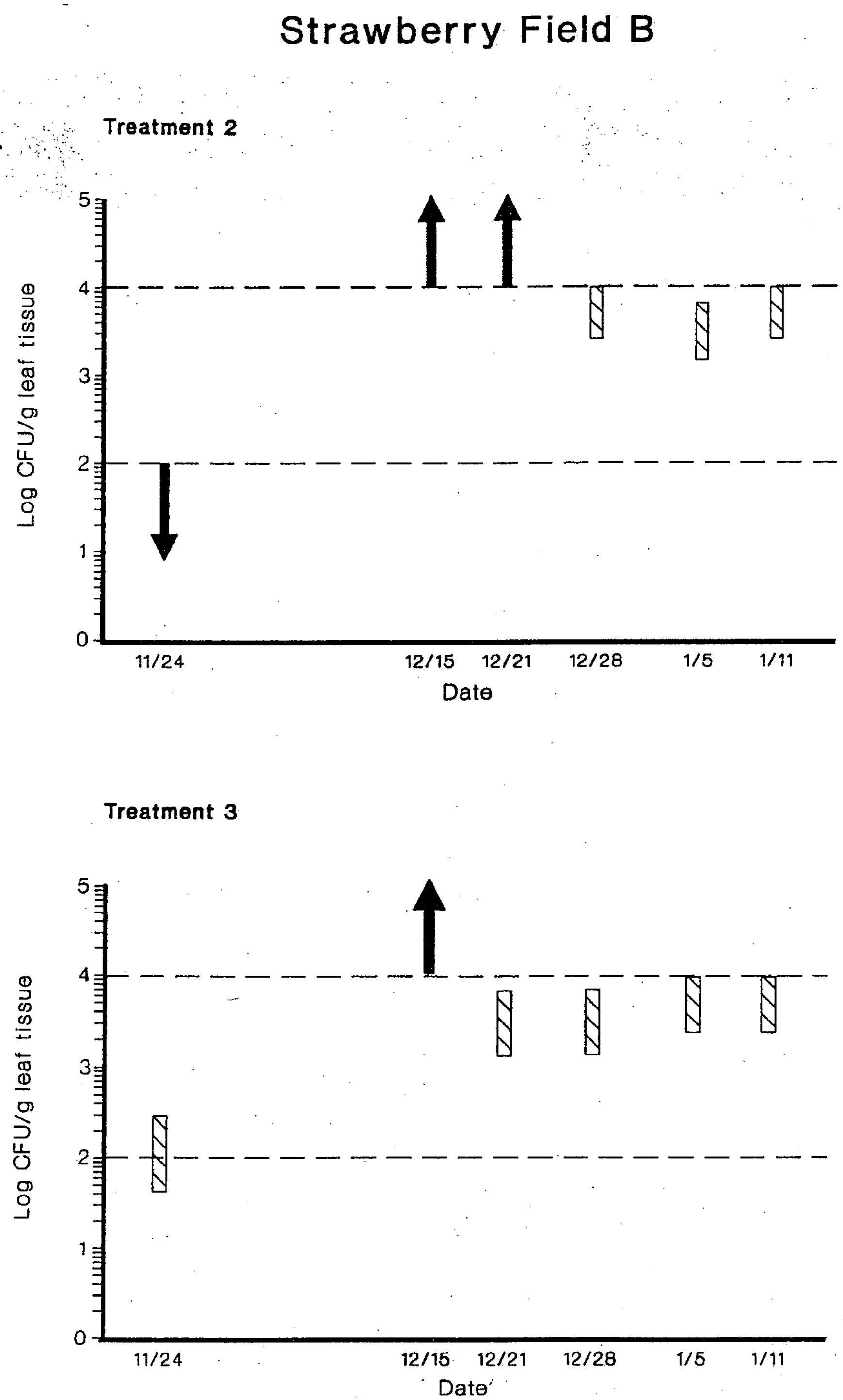

Figure 7. Calculated mean range of abundance of rifampicin resistant, fluorescent bacteria on strawberry leaflets collected from treatment plots during the Frostban® study. Frostban® treatment occurred 12/14/87. Solid arrows indicate values below or above the resolution of the isolation method. Hatched bars indicate the calculated mean range of abundance for bacteria detected between these upper and lower boundaries. Description of treatments 2 and 4 is given in Table 1.

While only one of the genetically engineered Pseudomonas species was applied to each treatment plot, both Frostban® bacteria could often be recovered from these plots (Appendix VI). Out of 40 samples taken from plots treated with a single Frostban® species, both species were recovered from 7 of these (17.5%). Genetically engineered Frostban® bacteria were also recovered from 5 out of 20 (25%) of the samples from control plots to which no genetically engineered bacteria were applied.

The abundance of total bacteria, as measured by the number of CFU on King's B medium amended with cycloheximide for background samples taken 22 days before pesticide application, varied between 10^2 and greater than 10^4 CFU/g leaf tissue for strawberry fields A and B, 10^2 and $10^{3.4}$ CFU/g leaf tissue for strawberry field C, and 10^2 and 10^4 CFU/g leaf tissue for off-site weeds (Appendix VII). Abundance of total bacteria in virtually all vegetation samples taken after application (235 out of 236 samples), was above the upper limit of resolution of 10^4 CFU/g leaf tissue.

Off-Site Samples - No genetically engineered bacteria were detected in off-site vegetation samples collected 22 days before or 1, 3, 7 or 28 days following microbial pesticide application (Appendix VII). Genetically engineered bacteria were recovered 14 and 22 days post-spray from vegetation collected off-site to the west and north of the experimental release site. During the four week sampling period following Frostban® application, genetically engineered bacteria were detected in 7 out of 141 (5%) off-site vegetation samples collected (Table 3).

Genetically engineered P. syringae and P. fluorescens were recovered 14 days post-spray from a perimeter strawberry sample and mixed weed sample, respectively, from

Table 3. Genetically engineered *Pseudomonas* species recovered from off-site vegetation sampled for the Winter, 1987, Frostban monitoring study.

Monitoring Area[a]	Distance in Meters from Treated Fields	Site	Days Post Spray	Frostban spp. Detected[b]
P	15	4	14	S
P	15	4	22	S
P	15	6	22	S
P	15	8	22	B
W	15	6	14	F
W	15	6	22	S
W	60	17	22	S

a. P= perimeter strawberry flat.
W= weed transect.

b. S= genetically-engineered *P. syringae*.
F= genetically-engineered *P. fluorescens*.
B= both genetically-engineered *Pseudomonas* spp.

an area just beyond the bare buffer zone to the west of the experimental release site. From this same area, just to the west of the buffer zone, recombinant bacteria were recovered 22 days post-spray from three perimeter strawberry samples. *Pseudomonas syringae* was detected alone in two of these perimeter strawberry samples and mixed with *P. fluorescens* in the third sample. Genetically engineered *P. syringae* was found in one of two replicate mixed weed samples collected 22 days post-spray from sampling locations 15 to 18 m to the west, and 60 m to the north, respectively, of the experimental strawberry site.

Rifampicin resistant fluorescent bacteria that were determined to be non-genetically engineered types could often be recovered from strawberry foliage from perimeter flats, vegetation from strawberry field C, and from mixed weed vegetation (Appendix VII).

Statistical analysis using a logistic regression model, indicated that there was a significant difference between dates ($p<.01$) and a nearly significant difference between areas ($p<.08$) for the presence of rifampicin resistant, non-genetically engineered fluorescent bacteria (Table 4). Logistic regression analysis indicated that the date x area interaction was not significant. The nearly significant difference between sampling areas appears to be due to the greater likelihood of finding antibiotic resistant, non-genetically engineered bacteria in weed samples, while the significant difference for date appears to be due to the high numbers of these bacteria on November 24 (background) and December 21, 1987 (7 days post-spray) relative to other sampling dates.

Quality Control Samples

All bacterial isolates of known identity submitted as laboratory quality control samples for gene probe analysis were correctly identified. When isolates from

Table 4. Analysis of variance results for presence of rifampicin resistant, non-genetically engineered bacteria using a logistic regression[a] model.

Source of Variation	Degree of Freedom	Chi-Square	Probability
Intercept	1	0.00	.98
Site	2	4.94	.08
Date	5	14.84	.01
Likelihood Ratio	10	9.68	.47

a. Logistic Model $\ln Px/qx = B_o + B_1 X_1 + B_2 X_2$

foliage samples, regardless of source, were identified as genetically engineered bacteria through gene probe analysis, test results in all cases (143 isolates) conformed to species identification based on additional diagnostic tests (Appendices VIII-IX).

Where genetically engineered Pseudomonas species were used to inoculate vegetation to create matrix spike samples (36 samples total), the species intentionally applied were always detected and correctly identified. However, on one sampling date (22 days post-spray), both species of Frostban® bacteria were recovered even though only genetically engineered species (P. syringae) had been applied to three quality control samples. Results from gene probe analysis for isolates from these three samples conformed to preliminary diagnostic test results indicating that contamination of these three quality control samples may have occurred during sample preparation. In any case, verification of the correct combination of genetically engineered Pseudomonas species applied to matrix spike samples occurred for 33 out of 36 of the samples (91.6%).

DISCUSSION

Genetically engineered bacteria were only recovered in air samples collected during Frostban® application along transects oriented in the direction of wind movement. Similarly, Frostban® bacteria were only detected in off-site vegetation samples collected west and north of the treated fields, also in the direction of wind movement. Thus, there is strong circumstantial evidence that the primary source of inoculum for off-site colonization of vegetation, was aerosol drift during Frostban® application. Genetically engineered bacteria were not recovered from off-site vegetation until 14 days post-spray. If Frostban® bacteria had been deposited off-site during pesticide application, between a one and 32o week period was required before these bacteria could be detected. However, it remains a possibility that off-site movement of genetically engineered bacteria occurred after application, and that a short period of time was necessary for bacterial multiplication before Frostban® bacteria could be detected.

Genetically engineered bacteria were primarily recovered in off-site vegetation between 15 and 18m from the western edge of the treated fields. Genetically engineered P. syringae was recovered from one sample 22 days post-spray at 60 m from the northern edge of strawberry field A. Although only one out of two samples yielded genetically engineered P. syringae from this northern site, and though relatively few rifampicin resistant fluorescent Pseudomonads, between 10^2 and $10^{2.7}$ bacteria/g leaf tissue, were recovered, this positive sample indicates the potential for movement of Frostban® bacteria either through drift or in air following deposition. Despite this apparent potential for movement, four weeks after Frostban® application no Frostban® bacteria could be detected in any off-site vegetation samples, although Frostban® bacteria were still consistently recovered from treatment plots. This indicates the transient nature of the colonization of genetically engineered bacteria on off-site vegetation.

Sixty meters was the farthest location from the experimental strawberry site where vegetation or air was sampled in this study. In future studies, when larger plots may be treated, it will be important to increase sampling of vegetation and air at distances greater than 60m from the experimental release site in order to further assess the potential for off-site movement and colonization by Frostban® bacteria.

The frequent recovery of genetically engineered P. syringae or P. fluorescens from experimental plots to which they were not applied indicates these bacteria can move short distances of 1.5m to 11.4m between positions in adjacent plots in drift during application or in air following deposition. Frostban® bacteria were detected in samples collected from treated and control plots one day after pesticide application. This is strong evidence that movement among plots of different treatment types occurred during Frostban® application due to drift of aerosol spray to nearby plots. However, it does not preclude the possibility that movement among plots of different treatment types following deposition also occurred subsequent to the spray event.

Frostban® bacteria were detected in air samples no further than 25 m north of strawberry field A, while off-site colonization of mixed weed vegetation occurred 60m north. To overcome problems encountered with the freezing of collection fluid in AGIs under ambient air temperature conditions of approximately 4°C in the field, intermittent air sampling was necessary. If it had been possible to sample air continuously over the entire application period, it is probable that at least an order of magnitude greater CFU would have been collected in each air sample. This would have increased the likelihood of detecting Frostban® bacteria at greater distances from the experimental release site where it is probable that they were at lower concentrations in air. Future environmental monitoring of microbial pesticides in air using a different air sampling approach which allows continuous air sampling should increase detection sensitivity in regions of lower bacterial cell concentration.

The presence of antibiotic resistant, fluorescent, non-genetically engineered bacteria in the environment may obscure the presence of genetically engineered bacteria or create additional work in processing environmental samples. The results of logistic regression analysis indicated that these antibiotic resistant bacteria were more likely to be detected on certain sampling dates and in weeds as compared with strawberry foliage.

There were two instances where relatively high levels of rifampicin resistant, fluorescent Pseudomonads were recovered without confirmation of the presence of engineered bacteria from strawberry plants in flats placed at the perimeter of the buffer zone. One week later, Frostban® bacteria were detected in these same locations. Strawberry plants in perimeter flats represented discrete, well defined sampling locations which could be serially sampled. It is possible that the presence of large numbers of rifampicin resistant, non-recombinant bacteria could have masked the presence of Frostban® bacteria which were subsequently detected in serial sampling.

The analytical method of gene probe analysis appeared to be a reliable diagnostic technique. Bacterial isolates from quality control samples were consistently identified correctly using gene probe analysis. Species identification through arginine dihydrolase and oxidase tests matched positive species identification through gene probe analysis in every case.

Cool, wet weather conditions during the sampling period following Frostban® application were conducive for growth and multiplication of test bacteria (Appendix X-XIII). Thus, environmental conditions provided an opportunity to evaluate the fate and persistence of genetically engineered bacteria under favorable circumstances.

While genetically engineered bacteria were recovered from air and off-site vegetation samples, these detections were limited to a relatively low percentage of total samples. The pattern of detection of Frostban® bacteria in samples of air and vegetation suggests that off-site movement of these bacteria was likely due to aerosol drift during microbial pesticide application.

REFERENCES

Lindow, S.E. 1982. Population dynamics of epiphytic ice nucleation active bacteria on frost sensitive plants and frost control by means of antagonistic bacteria. In Li, P.H. and Sakai, A., eds. Plant Cold Hardiness and Freezing Stress - Mechanisms and Crop Implications. Academic Press, N.Y., pp 395-416.

Lindow, S.E., Arny, D.C. and Upper, C.D. 1984. Bacterial ice nucleation: a factor in frost injury to plants. Plant Physiol. 70:1084-1089.

APPENDICES

APPENDIX I

Bacterial Media and Solutions

To prepare reagent stock solutions, 5.0 g of cycloheximide or 2.5 g of rifampicin were added to 50 ml 95% ethanol for cycloheximide and 100 ml DMSO for rifampicin.

King's B medium was prepared by adding 18.0 g of prepared media to 1000 ml of distilled water adding 10 ml of glycerol and steam sterilizing (121°C; 15 lbs.; 20 min.).

To prepare King's B medium with antibiotics, 1.5 ml stock cycloheximide and/or 3 ml stock rifampicin were added to King's B medium following steam sterilization. This amended media contained 150 ppm cycloheximide and 75 ppm rifampicin, or 150 ppm cycloheximide only, with approximately 15 ml media per culture plate. King's B medium amended with rifampicin was used within 7 to 10 days after preparation to avoid problems associated with antibiotic degradation.

Preparation of CuSO4 media, amended with cycloheximide and rifampicin, is similar to the above procedure with 0.798 g of anhydrous CuSO4 added prior to autoclaving (5 Mm CuSO4). A concentration of 5.0 Mm CuSO4 is believed to make the media selective for the copper resistant strain of _P_. _fluorescens_ applied by AGS.

The phosphate buffer (0.01M) was prepared by adding 1.74 g of potassium phosphate dibasic (Trihydrate) and 1.37 g of potassium phosphate monobasic to 1000 ml of distilled water, and adjusting the pH to 7.0.

The sodium-potassium peptone buffer was prepared by adding 10.95 g sodium phosphate dibasic heptahydrate, 3.5 g potassium phosphate monobasic, and 1.0 g Bacto peptone to 1000 ml of distilled water, and adjusting the pH to 7.1 and then steam sterilizing.

Nutrient broth amended with rifampicin was prepared by adding 8.0 g of Difco nutrient broth to 1000 ml of distilled water, steam sterilizing, and then adding 1 ml of rifampicin stock solution.

Luria broth media was made by adding 5 g of yeast, 10 g tryptone, and 5 g NaCl to 600 ml of deionized water. The pH was then adjusted to 7.5 with 1N NaOH and the volume increased to 930 ml with deionized water.

APPENDIX II

Oxidase Test

This test is particularly useful for differentiating pseudomonads from certain other Gram negative rods. The oxidase test is an indirect test for the presence of a cytochrome of the "C" type in the respiratory transport chain.

Required:
1. Filter paper.
2. 1.0% aqueous solution of N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride.
3. 24 hour bacterial culture.

Procedure:
1. Spread a loop of bacteria on a piece of filter paper soaked in 1.0% aqueous solution of N, N, N', N'-tetramethyl-p-phenylenediamine dihyrochloride. Production of a purple color in 5-10 seconds is considered a positive test; no color is a negative test.
2. *P. syringae* is oxidase negative and *P. fluorescens* is oxidase positive.

APPENDIX III

Arginine Dihydrolase Test

Arginine dihydrolase, present in certain bacteria, permits the conversion of arginine into ornithine, ammonia, and carbon dioxide with the production of ATP under anaerobic conditions.

Required:
1. 3 ml of Thornley's medium 2A.
2. 5 ml screw cap bottle.
3. 48 hour culture.

Procedures:
1. Pour about 3 ml media into 5 ml bottle.
2. Autoclave and let cool.
3. Stab inoculate the bacteria.
4. Seal the stab with sterile vaseline.
5. Incubate at room temperature for 3-4 days.
6. Note color change of the phenol red indicator. A pH change to the alkaline side (red) due to the ammonia is regarded as positive for arginine dihydrolase.
7. _P. syringae_ is arginine negative and _P. fluorescens_ arginine positive.

Reference:

1. Thornley, M. J. 1960. The differentiation of _Pseudomonas_ from other gram negative bacteria on the basis of arginine metabolism. J. Apl. Bacteriol. 23:37-52.

APPENDIX IV

Ice Nucleation Test

Ice nucleation is a characteristic of most wild type Pseudomonas bacteria. Frostban® bacteria lack the ability to act as a nucleus for ice formation.

Required:

1. Cooling chamber at -9°C.
2. Aluminum foil boats coated with xylene paraffin.
3. Culture to be tested.
4. Sterile toothpicks.
5. Dust free (covered) ELISA plates.
6. Multichannel automatic pipetter.

Procedures:

1. Lift cultures to be tested using a sterile toothpick.
2. Prepare a culture suspension in the well of the ELISA plate in 200 ul of sterile water, by using the toothpick as a disposable transfer tool.
3. Prepare a foil boat and float it on the cooling chamber.
4. Transfer a 20 ul drop of bacterial suspension to the cool foil using the automatic pipetter.
5. Allow 1-2 minutes for the droplets to freeze solid.
6. Check droplets with a sterile toothpick to determine if frozen.
7. About ten replicate droplets should be tested for each isolate to confirm or negate ice nucleation ability.

References:

1. Lindow, S. 1987. Personal communication.

APPENDIX V

Procedure for Isolation of Chromosomal DNA

. Overnight culture in Luria Broth (Appendix I)
. Pellet 5ml
. Wash in sterile dH20
. Resuspend cells in 3ml of 10% sucrose in 0.05M Tris pH 8
. Add 0.5ml of freshly made up lysozyme solution (10mg/ml in 0.25M Tris pH 8) and 1.2ml of 0.1M EDTA (pH 8)
. Leave on ice 10 min
. Add 0.6ml of 5% SDS (in H20) and vortex vigorously for 10 s
. Add 0.2mlof pancreatic RNase (1mg/ml)
. Incubate at 34 degrees C for 30 min
. Add 0.1ml proteinase K (1mg/ml)
. Incubate 60m at 34 degrees C
. Add 1/10vol NaOAc (pH 5.5 3M) and 2 vol of EtOH
. Collect DNA with a pasteur pipette
. Resuspend in 1ml TE
. Add 1/20 volume of spermidine 100mM
. Keep at room temp for 15 min
. Spin 10K 10m
. Remove supernatant
. Dissolve the pellet in 1ml of extraction buffer (70ml EtOH + 30ml 0.3M NaOAc pH 8, 10mM MgCl2)
. Extract spermidine during 1 hour, inverting occasionally
. Spin 10 min 10K
. Wash with 95% EtOH
. Dry under vacuum
. Resuspend in TE (10mM Tris; 1mK EDTA pH8)

Reference:
1. Chesney et. al. 1979, J. Mol. Biol. 130 p. 161-173)

APPENDIX VI

Genetically engineered *Pseudomonas* species applied and recovered from strawberry plants during the Frostban® Study. Date of microbial pesticide application was 12/14/87.

Experimental Strawberry Field	Site	Day Post Spray	Date	*Pseudomonas* spp. Applied by AGS[a]	*Pseudomonas* spp. Recovered by CDFA
A	2	-22	11/24/87	N	N
A	2	-22	11/24/87	N	N
A	2	1	12/15/87	S	S
A	2	1	12/15/87	S	S
A	2	7	12/21/87	S	S
A	2	7	12/21/87	S	S
A	2	14	12/28/87	S	S
A	2	14	12/28/87	S	S
A	2	22	01/05/88	S	S
A	2	22	01/05/88	S	S
A	2	28	01/11/88	S	S
A	2	28	01/11/88	S	S
A	4	-22	11/24/87	N	N
A	4	-22	11/24/87	N	N
A	4	1	12/15/87	F	F
A	4	1	12/15/87	F	F
A	4	7	12/21/87	F	F
A	4	7	12/21/87	F	F
A	4	14	12/28/87	F	B
A	4	14	12/28/87	F	F
A	4	22	01/05/88	F	B
A	4	22	01/05/88	F	B
A	4	28	01/11/88	F	B
A	4	28	01/11/88	F	F
A	6	-22	11/24/87	N	N
A	6	-22	11/24/87	N	N
A	6	1	12/15/87	N	N
A	6	1	12/15/87	N	S
A	6	7	12/21/87	N	N
A	6	7	12/21/87	N	N
A	6	14	12/28/87	N	B
A	6	14	12/28/87	N	N
A	6	22	01/05/88	N	F
A	6	22	01/05/88	N	N
A	6	28	01/11/88	N	N
A	6	28	01/11/88	N	N
B	1	-22	11/24/87	N	N
B	1	-22	11/24/87	N	N
B	1	1	2/15/87	N	N
B	1	1	12/15/87	N	N

Apppendix VI-2

Experimental Strawberry Field	Site	Day Post Spray	Date	Pseudomonas spp. Applied by AGS[a]	Pseudomonas spp. Recovered by CDFA
B	1	7	12/21/87	N	N
B	1	7	12/21/87	N	N
B	1	14	12/28/87	N	N
B	1	14	12/28/87	N	N
B	1	22	01/05/88	N	S
B	1	22	01/05/88	N	N
B	1	28	01/11/88	N	S
B	1	28	01/11/88	N	N
B	2	-22	11/24/87	N	N
B	2	-22	11/24/87	N	N
B	2	1	12/15/87	S	S
B	2	1	12/15/87	S	B
B	2	7	12/21/87	S	S
B	2	7	12/21/87	S	S
B	2	14	12/28/87	S	S
B	2	14	12/28/87	S	S
B	2	22	01/05/88	S	S
B	2	22	01/05/88	S	S
B	2	28	01/11/88	S	S
B	2	28	01/11/88	S	S
B	3	-22	11/24/87	N	N
B	3	-22	11/24/87	N	N
B	3	1	12/15/87	F	F
B	3	1	12/15/87	F	F
B	3	7	12/21/87	F	F
B	3	7	12/21/87	F	F
B	3	14	12/28/87	F	F
B	3	14	12/28/87	F	B
B	3	22	01/05/88	F	F
B	3	22	01/05/88	F	F
B	3	28	01/11/88	F	F
B	3	28	01/11/88	F	B

a. F= Genetically-engineered *P. fluorescens*
S= Genetically-engineered *P. syringae*
B= Both genetically engineered *Pseudomonas* species
N= No genetically engineered bacteria

Appendix VII. Diagnostic test results, final diagnoses and abundance of rifampicin resistant, fluorescent bacteria from field samples collected in the winter, 1987, Frostban Study.

AREA[a]	SITE	DAY POST SPRAY	RIF[b]	CYCLO[c]	FLUOR1	OXIDASE1	ARGININE1	ICE1	PROBE1	DIAGNOS1	FLUOR2	OXIDASE2	ARGININE2	ICE2	PROBE2	DIAGNOS2	FLUOR3	OXIDASE3	ARGININE3	ICE3	PROBE3	DIAGNOS3
A	2	22	0	2						N												
A	2	22	0	2						N												
A	2	1	4	4	+	-	-	-	S	S	+	+	+	-	N	N						
A	2	1	3	4	+	-	-	-	S	S	+	+	+	-	N	N						
A	2	7	4	4	+	-	-	-	S	S												
A	2	7	4	4	+	-	-	-	S	S												
A	2	14	3	4	+	-	-	-	S	S												
A	2	14	3	4	+	-	-	-	S	S												
A	2	22	3	4	+	-	-	-	S	S												
A	2	22	3	4	+	-	-	-	S	S												
A	2	28	3	4	+	-	-	-	S	S												
A	2	28	3	4	+	-	-	-	S	S												
A	4	22	1	2	?	-	-	-	N	N												
A	4	22	0	3						N												
A	4	1	4	4	+	+	+	-	F	F												
A	4	1	4	4	+	+	+	-	F	F												
A	4	7	4	4	+	+	+	-	F	F												
A	4	7	4	4	+	+	+	-	F	F												
A	4	14	3	4	+	-	-	-	S	S	+	+	+	-	F	F						
A	4	14	2	4	+	+	+	-	F	F												
A	4	22	3	4	+	-	-	-	S	S	+	+	+	-	F	F						
A	4	22	3	4	+	-	-	-	S	S	+	+	+	-	F	F						
A	4	28	3	4	+	-	-	-	S	S	+	+	+	-	F	F						
A	4	28	3	4	+	+	+	-	F	F												
A	6	22	0	2						N												
A	6	22	0	2						N												
A	6	1	0	4						N												
A	6	1	2	4	+	+	+	-	N	N	+	-	-	-	S	S						
A	6	7	0	4						N												
A	6	7	0	4						N												
A	6	14	2	4	+	-	-	-	S	S	+	+	+	-	F	F						
A	6	14	0	4						N												
A	6	22	1	4	+	+	+	-	F	F												
A	6	22	0	4						N												
A	6	28	0	4						N												
A	6	28	0	4						N												
B	1	22	1	2	?	-	-	-	N	N												
B	1	22	0	3						N												
B	1	1	0	4						N												

VII-2

AREA[a]	SITE	DAY POST SPRAY	RIF[b]	CYCLO[c]	FLUOR1	OXIDASE1	ARGININE1	ICE1	PROBE1	DIAGNOS1	FLUOR2	OXIDASE2	ARGININE2	ICE2	PROBE2	DIAGNOS2	FLUOR3	OXIDASE3	ARGININE3	ICE3	PROBE3	DIAGNOS3
B	1	1	1	4	+	+	+	-	N	N												
B	1	7	0	4						N												
B	1	7	0	4						N												
B	1	14	1	4	+	+	+	-	N	N												
B	1	14	0	4						N												
B	1	22	1	4	+	-	-	-	S	S												
B	1	22	0	4						N												
B	1	28	1	4	+	-	-	-	S	S												
B	1	28	0	4						N												
B	2	22	0	2						N												
B	2	22	0	1						N												
B	2	1	4	4	+	-	-	-	S	S	+	+	+	-	N	N						
B	2	1	4	4	+	-	-	-	S	S	+	+	+	-	F	F						
B	2	7	4	4	+	-	-	-	S	S												
B	2	7	4	4	+	-	-	-	S	S												
B	2	14	3	4	+	-	-	-	S	S												
B	2	14	3	4	+	-	-	-	S	S												
B	2	22	2	4	+	-	-	-	S	S												
B	2	22	3	4	+	-	-	-	S	S												
B	2	28	3	4	+	-	-	-	S	S												
B	2	28	3	4	+	-	-	-	S	S												
B	3	22	1	2	?	-	-	-	N	N												
B	3	22	0	2						N												
B	3	1	4	4	+	+	+	-	F	F												
B	3	1	4	4	+	+	+	-	F	F												
B	3	7	2	4	+	+	+	-	F	F												
B	3	7	3	4	+	+	+	-	F	F												
B	3	14	3	4	+	+	+	-	F	F												
B	3	14	2	4	+	-	-	-	S	S	+	+	+	-	F	F	+	+	+	-	F	F
B	3	22	3	4	+	+	+	-	F	F												
B	3	22	3	4	+	+	+	-	F	F												
B	3	28	3	4	+	+	+	-	F	F												
B	3	28	3	4	+	-	-	-	B	B	+	+	+	-	F	F						
O	7	22	1	2	?	-	-	+	N	N												
O	7	1	0	4						N												
O	7	7	0	4						N												
O	7	14	0	4						N												
O	7	22	0	4						N												
O	7	28	0	4						N												
O	8	22	0	2						N												

AREA[a]	SITE	DAY POST SPRAY	RIF[b]	CYCLO[c]	FLUOR1	OXIDASE1	ARGININE1	ICE1	PROBE1	DIAGNOS1	FLUOR2	OXIDASE2	ARGININE2	ICE2	PROBE2	DIAGNOS2	FLUOR3	OXIDASE3	ARGININE3	ICE3	PROBE3	DIAGNOS3
0	8	1	0	4						N												
0	8	7	1	4						N												
0	8	14	0	4						N												
0	8	22	0	4						N												
0	8	28	0	4						N												
0	9	22	1	2	?	-	-	-	N	N												
0	9	1	0	4						N												
0	9	7	0	4						N												
0	9	14	1	4	+	?	?	?	N	N												
0	9	22	0	4						N												
0	9	28	0	4						N												
0	10	22	0	3						N												
0	10	1	0	4						N												
0	10	7	0	4						N												
0	10	14	0	4						N												
0	10	22	0	4						N												
0	10	28	0	4						N												
0	11	22	0	2						N												
0	11	1	0	4						N												
0	11	7	0	4						N												
0	11	14	0	4						N												
0	11	22	0	4						N												
0	11	28	0	4						N												
0	12	22	1	2	?	-	-	-	N	N												
0	12	1	0	4						N												
0	12	7	0	4						N												
0	12	14	0	4						N												
0	13	22	0	2						N												
0	13	1	0	4						N												
0	13	7	0	4						N												
0	13	14	0	4						N												
0	13	22	0	4						N												
0	13	28	0	4						N												
0	14	22	0	2						N												
0	14	1	0	4						N												
0	14	7	0	4						N												
0	14	14	0	4						N												
0	15	22	0	1						N												
0	15	1	0	4						N												
0	15	7	0	4						N												

VII-4

AREA[a]	SITE	DAY POST SPRAY	RIF[b]	CYCLO[c]	FLUOR1	OXIDASE1	ARGININE1	ICE1	PROBE1	DIAGNOS1	FLUOR2	OXIDASE2	ARGININE2	ICE2	PROBE2	DIAGNOS2	FLUOR3	OXIDASE3	ARGININE3	ICE3	PROBE3	DIAGNOS3
O	15	14	0	4						N												
O	15	22	0	4						N												
O	15	28	0	4						N												
O	16	22	0	2						N												
P	1	22	0	3						N												
P	1	1	0	4						N												
P	1	1	1	4						N												
P	1	7	2	4						N												
P	1	14	0	4						N												
P	1	22	0	4						N												
P	1	28	0	4						N												
P	2	22	0	3						N												
P	2	1	0	4						N												
P	2	1	0	4						N												
P	2	7	0	4						N												
P	2	14	0	4						N												
P	2	22	0	4						N												
P	2	28	0	4						N												
P	3	22	1	2	?	-	-	-	N	N												
P	3	1	0	4						N												
P	3	1	1	4						N												
P	3	7	0	4						N												
P	3	14	0	4						N												
P	3	22	0	4						N												
P	3	28	0	4						N												
P	4	22	0	3						N												
P	4	1	0	4						N												
P	4	1	0	4						N												
P	4	7	3	4						N												
P	4	14	3	4	+	-	-	-	S	S												
P	4	22	1	4	+	-	-	-	S	S												
P	4	28	0	4						N												
P	5	22	0	3						N												
P	5	1	0	4						N												
P	5	1	0	4						N												
P	5	7	0	4					N	N												
P	5	14	0	4						N												
P	5	22	0	4						N												
P	5	28	0	4						N												
P	6	22	0	4						N												

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AREA[a]	SITE	DAY POST SPRAY	RIF[b]	CYCLO[c]	FLUOR1	OXIDASE1	ARGININE1	ICE1	PROBE1	DIAGNOS1	FLUOR2	OXIDASE2	ARGININE2	ICE2	PROBE2	DIAGNOS2	FLUOR3	OXIDASE3	ARGININE3	ICE3	PROBE3	DIAGNOS3
P	6	1	0	4						N												
P	6	1	0	4						N												
P	6	7	0	4						N												
P	6	14	0	4						N												
P	6	22	1	4	+	-	-	-	S	S												
P	6	28	0	4						N												
P	7	22	0	4						N												
P	7	1	0	4						N												
P	7	1	0	4						N												
P	7	7	2	4						N												
P	7	14	0	4						N												
P	7	22	0	4						N												
P	7	28	0	4						N												
P	8	22	0	2						N												
P	8	1	0	4						N												
P	8	1	0	4						N												
P	8	7	2	4						N												
P	8	14	0	4						N												
P	8	22	3	4	+	-	-	-	S	S	+	+	+	-	F	F						
P	8	28	0	4						N												
P	9	22	0	4						N												
P	9	1	0	4						N												
P	9	1	1	4						N												
P	9	7	0	4						N												
P	9	14	0	4						N												
P	9	22	0	4						N												
P	9	28	0	4						N												
P	10	22	0	2						N												
P	10	1	0	3						N												
P	10	1	0	4						N												
P	10	7	0	4						N												
P	10	14	0	4						N												
P	10	22	0	4						N												
P	10	28	0	4						N												
P	11	22	1	3	?	-	-	-	N	N												
P	11	1	0	4						N												
P	11	1	0	4						N												
P	11	7	0	4						N												
P	11	14	0	4						N												
P	11	22	0	4						N												

A[a]REA	SITE	DAY POST SPRAY	R[b]IF	C[c]YCLO	FLUOR1	OXIDASE1	ARGININE1	ICE1	PROBE1	DIAGNOS1	FLUOR2	OXIDASE2	ARGININE2	ICE2	PROBE2	DIAGNOS2	FLUOR3	OXIDASE3	ARGININE3	ICE3	PROBE3	DIAGNOS3
P	11	28	0	4						N												
P	12	22	0	4						N												
P	12	1	0	4						N												
P	12	1	0	4						N												
P	12	14	0	4						N												
P	12	22	0	4						N												
P	12	28	0	4						N												
P	13	22	0	2						N												
P	13	1	0	4						N												
P	13	1	0	4						N												
P	13	7	0	4						N												
P	13	14	0	4						N												
P	13	22	0	4						N												
P	13	28	0	4						N												
P	14	22	0	2						N												
P	14	1	0	4						N												
P	14	1	0	4						N												
P	15	22	1	4	?	-	-	-	N	N												
P	15	1	0	4						N												
P	15	1	0	4						N												
P	15	7	0	4						N												
P	15	14	0	4						N												
P	15	22	3	4						N												
P	15	28	0	4						N												
P	16	22	0	4						N												
P	16	1	0	4						N												
P	16	1	1	4						N												
P	16	7	0	4						N												
P	16	14	3	4	+	+	+	-	N	N												
P	16	22	0	4						N												
P	16	28	0	4						N												
W	1	22	0	2						N												
W	1	1	0	4						N												
W	1	7	2	4						N												
W	1	14	0	4						N												
W	1	22	0	4						N												
W	1	28	0	4						N												
W	2	22	0	4						N												
W	2	1	0	4						N												
W	2	7	0	1						N												

AREA[a]	SITE	DAY POST SPRAY	RIF[b]	CYCLO[c]	FLUOR1	OXIDASE1	ARGININE1	ICE1	PROBE1	DIAGNOS1	FLUOR2	OXIDASE2	ARGININE2	ICE2	PROBE2	DIAGNOS2	FLUOR3	OXIDASE3	ARGININE3	ICE3	PROBE3	DIAGNOS3
W	2	7	0	4						N												
W	2	14	0	4						N												
W	2	14	0	4						N												
W	2	22	0	4						N												
W	2	22	0	4						N												
W	2	28	0	4						N												
W	2	28	1	4	+	+	+	-	N	N												
W	3	22	0	4						N												
W	3	1	0	4						N												
W	3	7	2	4						N												
W	3	14	0	4						N												
W	3	22	0	4						N												
W	3	28	0	4						N												
W	4	22	0	4						N												
W	4	1	0	4						N												
W	4	7	0	4	-				N	N												
W	4	14	3	4						N												
W	4	22	0	4						N												
W	4	28	0	4						N												
W	5	22	1	4						N												
W	5	1	0	4						N												
W	5	7	1	4						N												
W	5	14	0	4						N												
W	5	22	0	4						N												
W	5	28	0	4						N												
W	6	22	1	2	?	-	-	-	N	N												
W	6	1	0	4						N												
W	6	7	2	4						N												
W	6	14	1	4	+	?	?	-	F	F	+	+	+	-	F	F						
W	6	22	1	4	+	-	-	-	S	S												
W	6	22	0	4						N												
W	6	28	0	4						N												
W	6	28	0	4						N												
W	17	1	0	4						N												
W	17	7	0	4						N												
W	17	14	0	4						N												
W	17	22	0	4						N												
W	17	22	1	4	+	-	-	-	S	S												

AREA[a]	SITE	DAY POST SPRAY	RIF[b]	CYCLO[c]	FLUOR1	OXIDASE1	ARGININE1	ICE1	PROBE1	DIAGNOS1	FLUOR2	OXIDASE2	ARGININE2	ICE2	PROBE2	DIAGNOS2	FLUOR3	OXIDASE3	ARGININE3	ICE3	PROBE3	DIAGNOS3
W	17	28	1	4	+	-	-	-	N	N												
W	17	28	0	4						N												

a. A= Strawberry field A
B= Strawberry field B
O= Strawberry field C
P= Perimeter strawberry flats
W= Offsite weeds

b. RIF= Abundance of fluorescent bacteria on Kings B medium amended with rifampicin.

c. Cyclo= Abundance of fluorescent bacteria in Kings B medium. The following rating scheme was used for abundance determination for culture plates:
O= no colonies present; 1= 1-5 colonies present; 2= 6-25 colonies; 3= 26-100 colonies; 4= greater than 100 colonies.
Diagnostic tests:
Fluor= fluorescence test; Oxidase= oxidase test; Arginine= arginine dihydrolase test; Ice= ice nucleation test;
Probe= gene probe analysis results; Diagnosis= final diagnosis by CDFA Plant Pathology Lab.
+= positive; -= negative; ?= uncertain. Number after diagnostic test refers to isolate tested.

Appendix VIII. Diagnostic test results and final diagnoses for quality control samples submitted for analysis for the Winter 1987, Frostban Monitoring Study.

			Diagnostic Test Results and Final Diagnoses [c]																							
QCSOURCE [a]	QCID [b]	DAY POST SPRAY	FLUOR1	OXID1	ARGIN1	ICE1	PROBE1	DIAGNO1	FLUOR2	OXID2	ARGIN2	ICE2	PROBE2	DIAGNO2	FLUOR3	OXID3	ARGIN3	ICE3	PROBE3	DIAGNO3	FLUOR4	OXID4	ARGIN4	ICE4	PROBE4	DIAGNO4
1	B	22	+	-	-	-	S	S	+	+	+	-	F	F	+	+	+	-	F	F	+	+	+	-	F	F
1	F	22	+	+	+	-	F	F	+	+	+	-	F	F	+	+	+	-	F	F	+	+	+	-	F	F
1	S	22	+	-	-	-	S	S																		
1	F	22	+	+	+	-	F	F	+	+	+	-	F	F	+	+	+	-	F	F	+	+	+	-	F	F
1	S	22	+	-	-	-	S	S																		
1	B	22	+	-	-	-	S	S	+	+	+	-	F	F	+	+	+	-	F	F	+	+	+	-	F	F
1	S	1	+	-	-	-	S	S																		
1	F	1	+	+	+	-	F	F																		
1	S	1	+	-	-	-	S	S																		
1	F	1	+	+	+	-	F	F																		
1	F	1	+	+	+	-	F	F																		
1	S	1	+	-	-	-	S	S																		
1	F	7	+	+	+	-	F	F																		
1	S	7	+	-	-	-	S	S																		
1	S	7	+	-	-	-	S	S																		
1	F	7	+	+	+	-	F	F																		
1	S	7	+	-	-	-	S	S																		
1	F	7	+	+	+	-	F	F																		
1	S	14	+	-	-	-	S	S																		
1	F	14	+	+	+	-	F	F																		
1	S	14	+	-	-	-	S	S																		
1	F	14	+	+	+	-	F	F																		
1	S	14	+	-	-	-	S	S																		
1	F	14	+	+	+	-	F	F																		
1	F	22	+	+	+	-	F	F																		
1	F	22	+	+	+	-	F	F																		
1	F	22	+	+	+	-	F	F	+	+	+	-	F	F												
1	S	22	+	-	-	-	S	S	+	+	+	-	F	F												
1	S	22	+	-	-	-	S	S	+	+	+	-	F	F	+	+	+	-	F	F						
1	S	22	+	-	-	-	S	S	+	+	+	-	F	F												
1	F	28	+	+	+	-	F	F																		
1	S	28	+	-	-	-	S	S																		
1	S	28	+	-	-	-	S	S																		
1	S	28	+	-	-	-	S	S																		
1	F	28	+	+	+	-	F	F	+	+	+	-	F	F												
1	F	28	+	+	+	-	F	F	+	+	+	-	F	F												
2	F	22	+	+	+	-	F	F																		
2	N	22	+	+	+	?	N	N																		
2	S	22	+	-	-	-	S	S																		
2	N	1	+	-	-	?	N	N																		
2	F	1	+	+	+	-	F	F																		

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QCSOURCE[a]	QCID[b]	DAY POST SPRAY	FLUOR1	OXID1	ARGIN1	ICE1	PROBE1	DIAGNO1	FLUOR2	OXID2	ARGIN2	ICE2	PROBE2	DIAGNO2	FLUOR3	OXID3	ARGIN3	ICE3	PROBE3	DIAGNO3	FLUOR4	OXID4	ARGIN4	ICE4	PROBE4	DIAGNO4
			Diagnostic Test Results and Final Diagnoses[c]																							
2	N	1	?	+	+	?	N	N																		
2	S	1	+	-	-	-	S	S																		
2	F	7	+	+	+	-	F	F																		
2	N	7					N	N																		
2	N	7					N	N																		
2	S	7	+	-	-	-	S	S																		
2	N	14	+	+	+	?	N	N																		
2	S	14	+	-	-	-	S	S																		
2	N	14	+	?	+	?	N	N																		
2	F	14	+	+	+	-	F	F																		
2	N	22	+	+	+	?	N	N																		
2	S	22	+	-	-	-	S	S																		
2	F	22	+	+	+	-	F	F																		
2	N	22	+	+	+	?	N	N																		
2	S	28	+	-	-	-	S	S																		
2	F	28	+	+	+	-	F	F																		
2	N	28	+	+	+	?	N	N																		
3	S	22	+	-	-	-	S	S																		
3	N	22	+	+	+	-	N	N																		
3	F	22	+	+	+	-	F	F																		
3	S	1	+	-	-	-	S	S																		
3	F	1	+	+	+	-	F	F																		
3	F	1	+	+	+	-	F	F																		
3	N	1	+	+	+	-	N	N																		
3	S	1	+	-	-	-	S	S																		
3	F	7	+	+	+	-	F	F																		
3	N	7	+	+	+	-	N	N																		
3	N	7	+	+	+	-	N	N																		
3	F	7	+	+	+	-	F	F																		
3	S	7	+	-	-	-	S	S																		
3	S	7	+	-	-	-	S	S																		
3	F	14	+	+	+	-	F	F																		
3	S	14	+	-	-	-	S	S																		
3	N	14					N	N																		
3	F	14	+	+	+	-	F	F																		
3	N	14	+	+	+	-	N	N																		

Diagnostic Test Results and Final Diagnoses [c]

QCSOURCE[a]	QCID[b]	DAY POST SPRAY	FLUOR1	OXID1	ARGIN1	ICE1	PROBE1	DIAGNO1	FLUOR2	OXID2	ARGIN2	ICE2	PROBE2	DIAGNO2	FLUOR3	OXID3	ARGIN3	ICE3	PROBE3	DIAGNO3	FLUOR4	OXID4	ARGIN4	ICE4	PROBE4	DIAGNO4
3	N	14	+	+	+	-	N	N																		
3	N	28	+	+	+	?	N	N																		

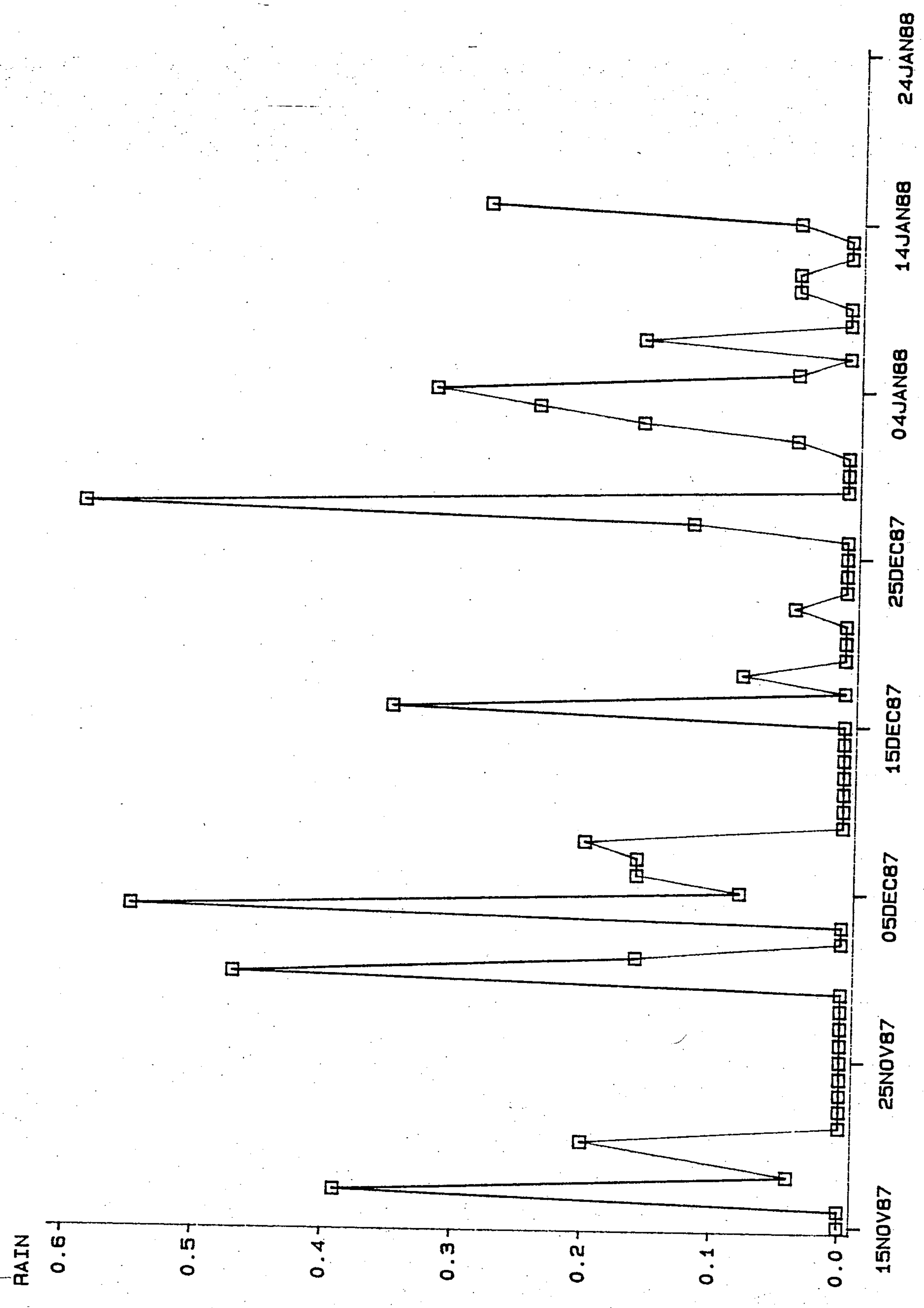
a. 1= Spiked strawberry foliage quality control samples; 2= laboratory isolates from BCSP to UCB via A&I; 3= laboratory isolates from A&I to UCB.
b. Identity of bacteria applied to foliage or submitted as control cultures.
F= Control cultures
S= Genetically-engineered *P. fluorescens*
B= Both genetically-engineered *Pseudomonas* species
N= No genetically-engineered bacteria
c. Diagnostic tests:
Fluore = Fluorescence test
Oxid = Oxidase test
Argin = Arginine test
Ice = Ice nucleation test
Probe = Gene probe test results
Diagno = Final diagnosis by CDFA Plant Pathology lab
\+ = positive
\- = negative
? = uncertain
Number after test refers to isolate tested.

APPENDIX IX

Diagnostic Test Profile for Genetically-Engineered
Pseudomonas Species

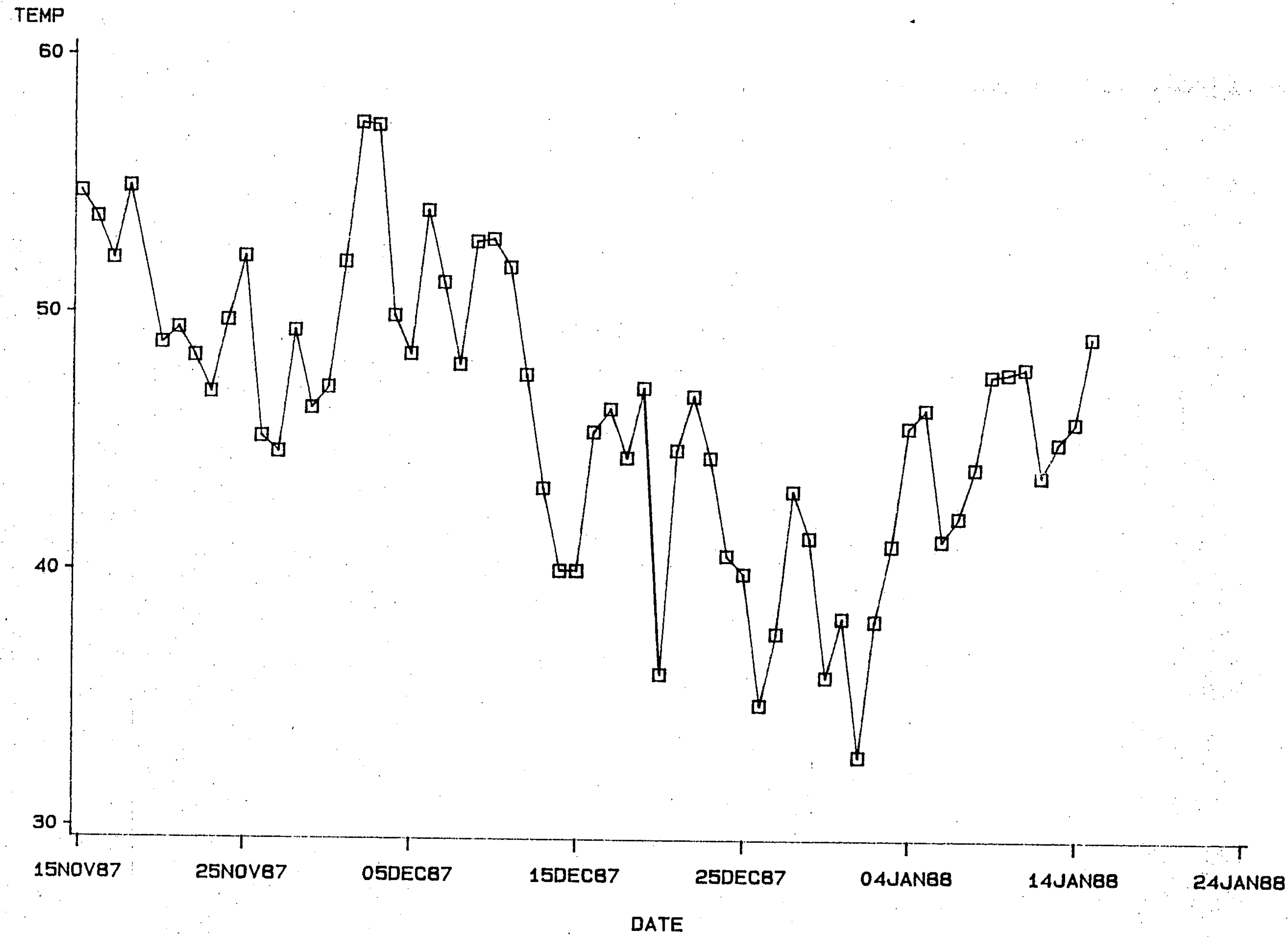
	P. syringae	*P. fluorescens*
Fluorescence	+	+
Oxidase	-	+
Arginine Dihydrolase	-	+
Ice nucleation	-	-

APPENDIX X



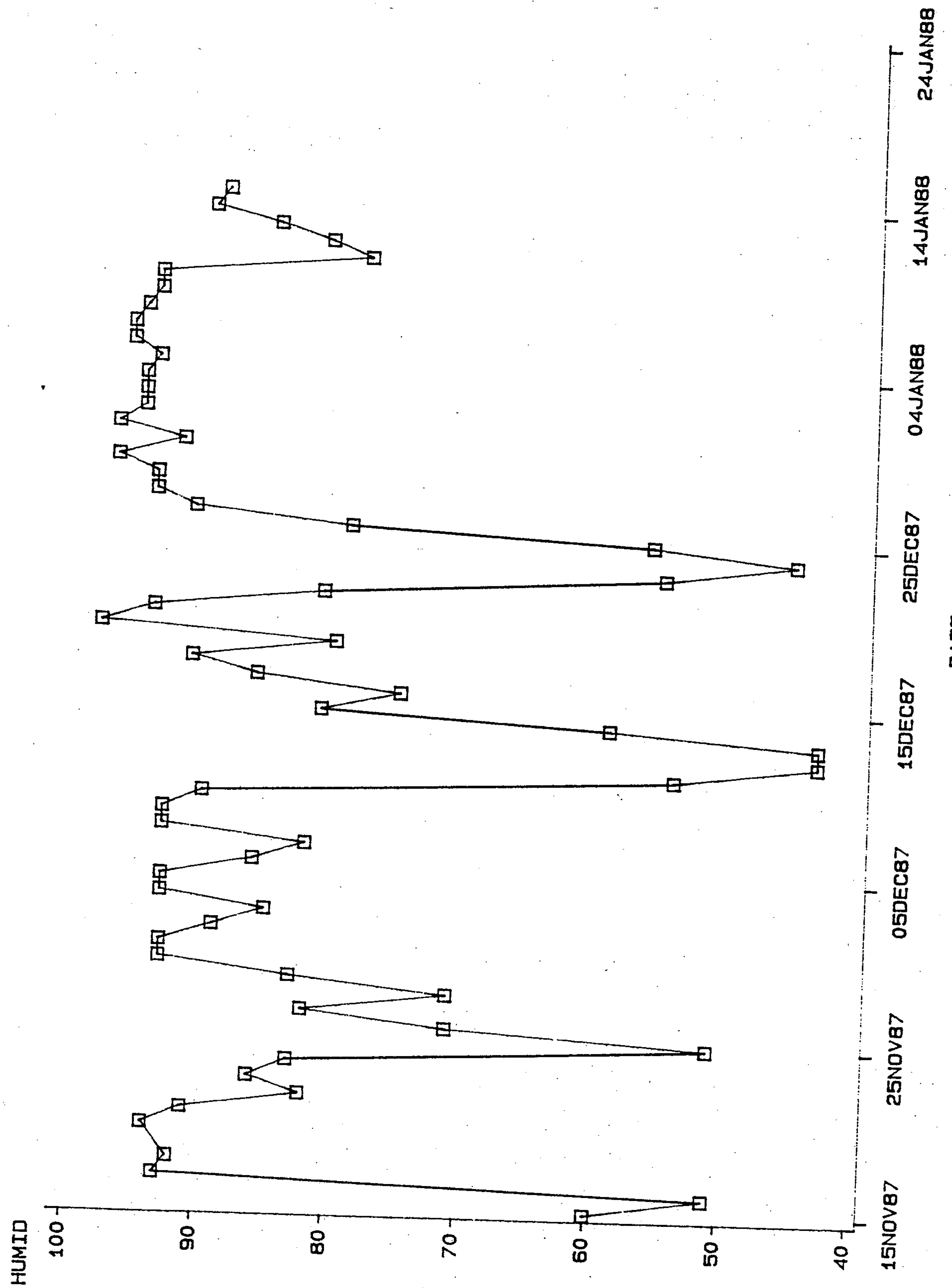
Total daily rainfall (inches) recorded in Brentwood, California, from November 15, 1987 to January 25, 1988

APPENDIX XI



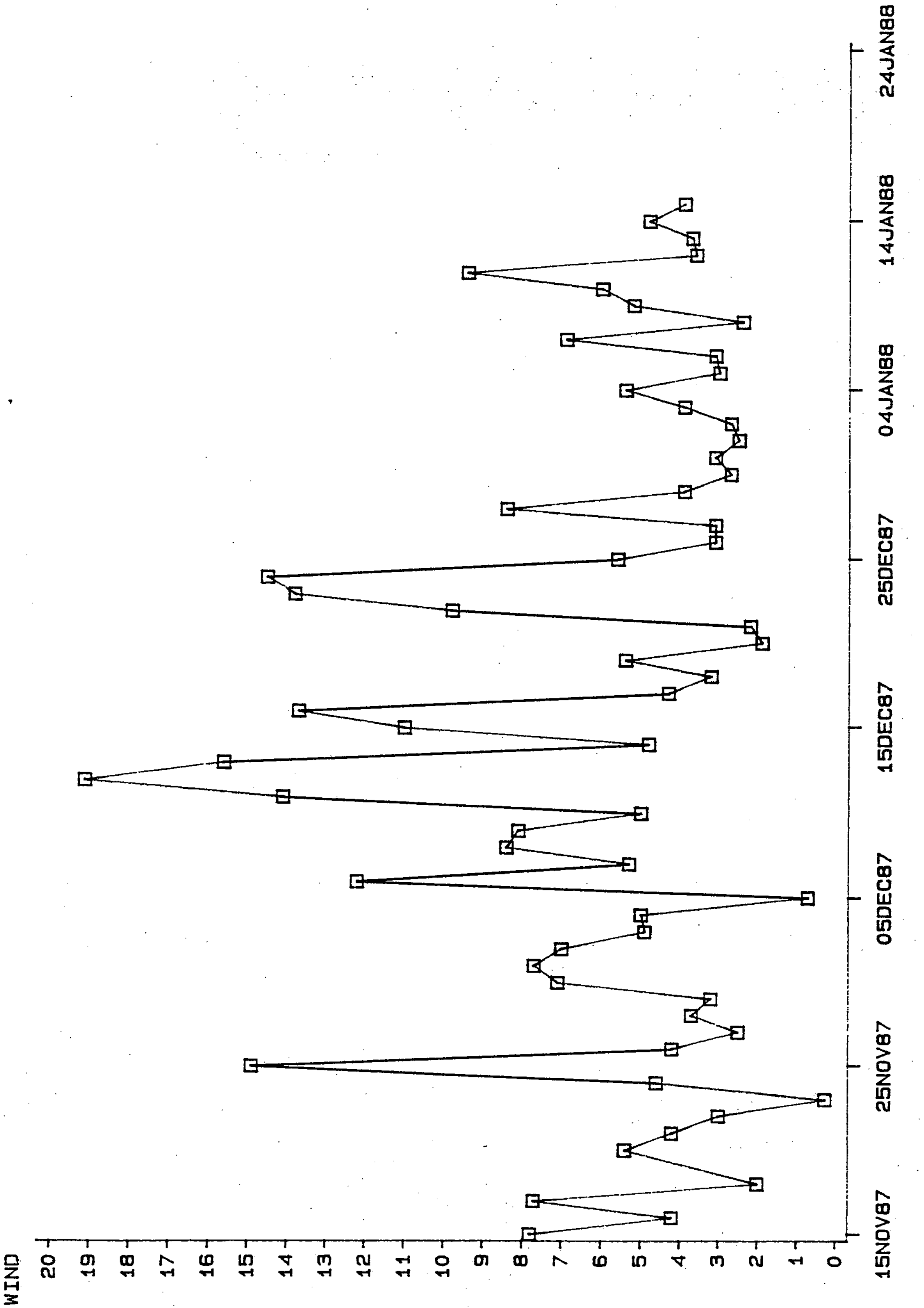
Average daily temperature (^{O}F) recorded in Brentwood, California, from November 15, 1987 to January 15, 1988

APPENDIX XII



Average daily humidity (x percent) recorded in Brentwood, California, from NOvember 15, 1987 to January 15, 1988

APPENDIX XIII



Average daily wind speed (mph) recorded in Brentwood, California, from November 15, 1987 to January 15, 1988